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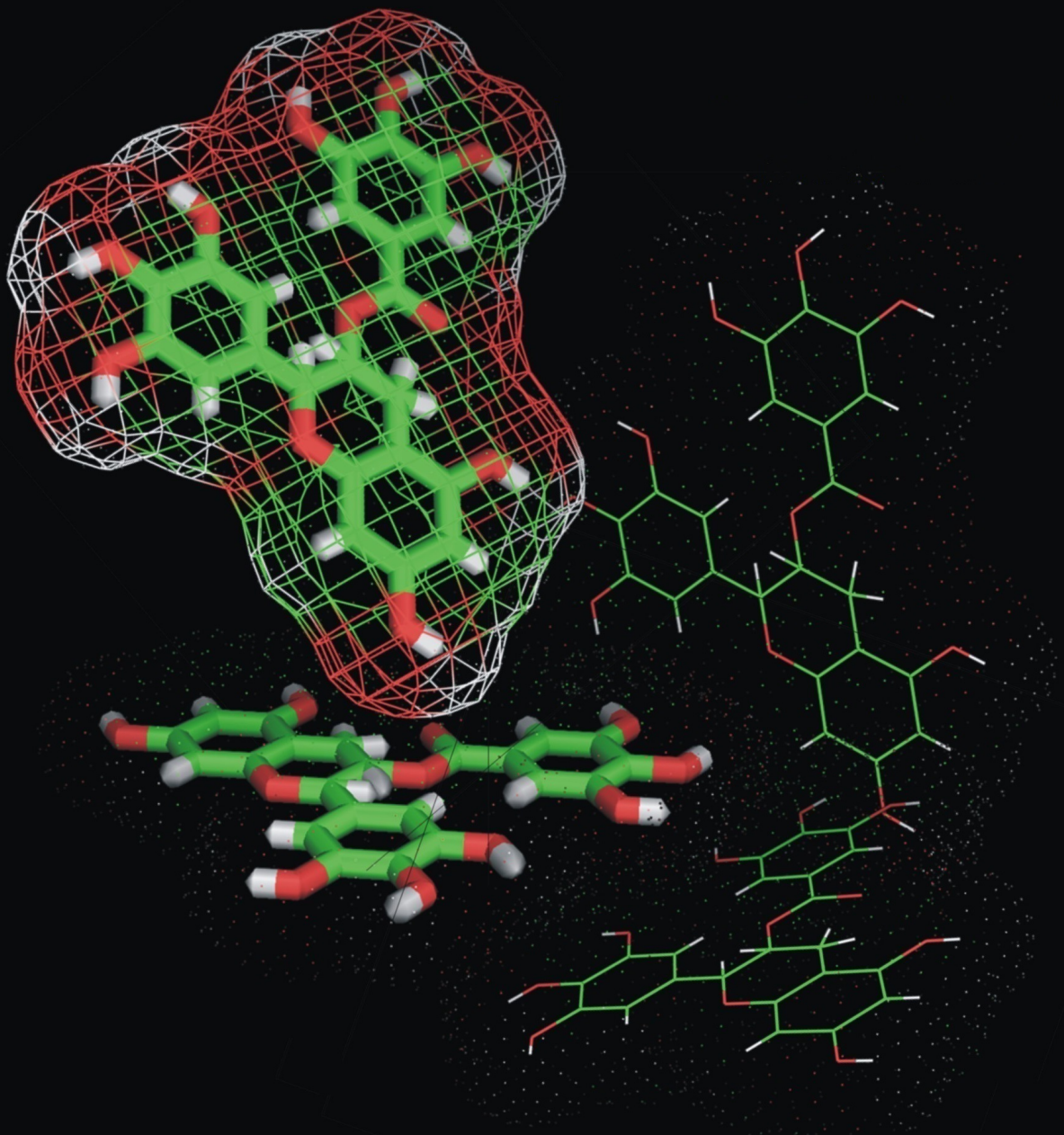

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Table of Content

Molecular Detection, Histopathology, and Scanning Electron Microscopy of *Myxobolus koi* Infecting *Cyprinus carpio* Koi

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Abstract

Myxobolus koi is a type of parasite that infects many freshwater fish through myxospores, resulting in a disease called myxobolosis. This research aimed is to investigate the clinical symptoms that occur in the gills of infected Koi carp (*C. carpio*). The utilized methods in this study are molecular detection, histopathology, and Scanning Electron Microscopy (SEM). Koi carp samples that are suspected to be infected with *Myxobolus koi* show several clinical signs, such as swollen and pale-colored gills and gill covers (opercula) that do not close completely, as well as white spots that appear on the edge of the lamella. The results of this study showed that through histological observations, there were changes characterized by hyperplasia of the primary lamella cartilage that envelops parasitic cysts and the encapsulation process that surrounds the cysts by gill cartilage in infected fish; SEM showed oval and elongated spores of a size of ± 12 µm (800x). The DNA template from gill tissues in clinically infected and uninfected fish was examined by PCR testing with primers ERB 1 (Forward) and ERB 10 (Reverse); the results of electrophoresis in infected fish were detected at 2000 bp.

Keywords: *Cyprinus carpio*, Histopathology, Molecular Detection, *Myxobolus koi*, Parasite, SEM.

INTRODUCTION

Globally, the aquaculture industry in the ornamental fish sector has grown rapidly in recent decades [1,2]. The value of global trade in ornamental fish estimated at more than 15 million dollars, with an annual growth of 8%. One of these ornamental fish is koi fish, which is an ornamental strain of carp (*Cyprinus carpio*) [3,4].

With the increasing demand for trade in ornamental fish, there is an increased risk of cross-sectional spread of several pathogens. Trade in the ornamental fish industry has indirectly moved millions of fish every year around the world, in some cases resulting in myxozoan parasites moving to new areas that cause pathological changes and death among fish [5]. Myxozoan parasites are a type of cnidarian endoparasites that are widely distributed throughout the world and cause economic losses in fisheries and aquaculture. Deaths from *Myxobolus koi* have also been reported in koi that transferred from Asia to the United Kingdom and the United States [6]. *Myxobolus koi is* a type of myxozoan parasite that infects many freshwater fish, resulting in a disease called myxobolosis. Myxobolosis that infect the gills of carp in the juvenile stage results in large losses [7,8]. At present, there are 29 *Myxobolus* species known as parasites of *C. carpio*, of which 17 infect the gills [9]. Myxozoans are among the most abundant parasites in nature [10,11]. Their life cycles involve two hosts: an invertebrate, usually an annelid, and a vertebrate, usually a fish [12,13]. They affect fish species in their natural habitats but also constitute a menace for fish aquaculture [14,17].

Gill myxobolosis due to *Myxobolus koi* (Myxozoa; Myxosporea) (Kudo, 1920) of common carp (*Cyprinus carpio*) juveniles have been known to occur occasionally in Japanese carp culture and cause serious damage to fish farmers in 1978 [7]. Attacks of *Myxobolus* found in Indonesia in 1974 and 1978, which caused the death of up to 100% of koi fish, especially in the juvenile phase [18].

In 2013, *Myxobolus koi* was included in the list of Class I Quarantine of Fish Pests and Diseases in the parasite type by Ministry of Fishery and Maritime Affairs Decision Number 26/Kepmen-Kp/2013 on the Establishment of Quarantine of Fish Pest and Disease Types, Groups, Carrier Media, and Their Distribution, which means that if this kind of parasite is found in carrier media, destruction must be carried out. [19]. Fish diseases are usually difficult to control and cure; when the disease infection has taken place, it is often late for its management and the prevention of greater losses. It becomes very important to find out the health level of fish [20].

Clinical parameters and zootechnical indices have been regarded as insufficient to monitor fish health during early infection. Therefore, in addition to traditional markers (biochemistry,

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histology, morphology, and physiology), it is important to look for alternative parameters such as molecular biomarkers [21,22,23]. The genome strategy is revolutionizing scientific research in understanding fish physiology and gene evolution because it is relatively easy to isolate novel genes and homologs using public databases [24,25]. The aim of this research is to investigate the clinical symptoms that occur in the gills of Koi carp (*C. carpio*) infected by *Myxobolus* sp. The utilized methods are traditional methods (histopathology, SEM) as well as the molecular detection method, which are seen as the most representative and effective methods for checking the health level of fish.

MATERIALS AND METHODS

Sampling

Juvenile koi (*Cyprinus carpio*) of approximately 5-7 cm in length and 8-10 grams in weight were obtained from ponds in Kemloko and Kedungwaru Villages of Nglegok Sub-District in Blitar, Province of East Java. Eight samples were collected from December 2018 to January 2019. The live fish were transported to the Bioscience Laboratory of Brawijaya University and the Molecular Biology Laboratory of the Class I Fish Quarantine and Inspection Agency of Surabaya.

For Scanning Electron Microscopy and molecular identification, taking gills of the test fish and preserved in 95% ethanol solution steps was conducted according to the protocol [26,27]. Fish gills containing plasmids were fixed with Davidson solution, gradient-dehydrated, embedded in paraffin wax and sectioned at 3-5 µm, and given color with hematoxylin and eosin [28]. Ethanol-preserved plasmids were used for genomic DNA extraction according to the procedure recommended by the manufacturer [29].

Histopathology Study

Making of Histological Block Tissue

1. Specimens were fixed using Davidson solution for a maximum of 24 hours and then transferred to a 10% formalin buffer solution.
2. Samples were cut into small pieces measuring ± 1 cm. The samples were inserted into a cassette or specimen container and labeled according to the sample number.
3. These were inserted into a tissue processor container and the device was programmed accordingly.
4. The dehydration process used multilevel alcohol solutions ranging from 70%, 80% (2x replications), to 85%, each for 2 hours. Subsequent processing used pure alcohol, conducted 3 times each for two hours.
5. The clearing process used xylene, with 3 replications each for 30 minutes.
6. The embedding process utilized liquid paraffin at a temperature of 58°C, with 2 replications each for 2 hours.
7. Blocking was performed by removing the specimens from the cassette to be printed using a paraffin mold, then the block was put in the freezer for 5 minutes. The block was removed from the mold and trimmed by forming a 1.5 cm square.
8. By using a microtome, the tissue was cut to a thickness of 3-5 µ and immediately floated in a water bath filled with distilled water heated to 50°C. Paraffin tape was removed using a glass, and the object was air-dried and labeled.

Coloring of Histological Tissue

1. The tissue pieces that have been attached to the glass are arranged in a staining jar and then inserted into an incubator at 37°C for 1 hour, and the glass is dried.
2. The deproteination process was carried out using a xylene solution with 2 repetitions each for 5 minutes.
3. Dehydration was performed using alcohol starting from pure alcohol to 95% alcohol, with 2 repetitions 10 times for each dye, or ±1.
4. Rinsing was performed 10 times with distilled water.
5. Next, the coloring process used Hematoxylin dye for 2 minutes, before rinsing with running water for 5 minutes and followed by eosin staining for 10 minutes.
6. The dehydration process used alcohol starting from pure alcohol to 95% alcohol.
7. The clearing process used xylene, with 2 repetitions for 10 times or ± 1 minute.
8. For mounting, slides containing pieces of tissue were removed from the staining jar one by one and then covered with a glass cover that had been given an Entellan solution.
9. Tissue preparations were then observed under a microscope with 40x magnification for analysis.
10. Existing tissue abnormalities were observed [26,28].

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Scanning Electron Microscopy

For SEM analysis of the myxozoan parasites, 2.5% glutaraldehyde fixation solution was administered for two hours at 4 °C, followed by dehydration with ethanol and air-drying. The sample was then coated with metallic platinum in an IB-2 ion counter and examined with a ZEISS Scanning Electron Microscope at an accelerating voltage of 15.00 KV with 10.00 KX magnification. The host fish was examined and fixed in 2.5% glutaraldehyde solution for two hours at 4 °C, followed by dehydration with ethanol and rinsing with pure acetone and mixtures with amyl acetate in 3:1, 2:2, and 1:3 ratios, and finally with 100% amyl acetate. The tissues were then dried at critical point using CO_2 in a HCP:2 Critical Point Dryer (Hitachi), coated with metallic gold at accelerating voltages of 15 and 20 KV [26,27].

DNA Extraction

DNA was extracted using the Silica Extraction Kit (Gene) from tissues preserved in pure ethanol solution. Each gill and intestinal tissue sample identified as Myxobolus-infected or healthy was inserted into 1.5 mL microtubes, to which 900 µl GT Buffer is added before being mashed with a grinding pestle and centrifuged at a speed of 12000 rpm for 3 minutes. The 600 µl layers were put into new 1.5 mL microtubes, given 40 µL of silica, vortexed until homogeneous, and centrifuged at a speed of 12000 rpm for 15 seconds (no more than 20 seconds). After centrifuging, the solution was discarded and the silica pellets were washed with 500 µL GT Buffer, vortexed until a suspension was formed, and centrifuged at a speed of 12000 rpm for 15 seconds (no more than 20 seconds). The solution was discarded again and 1 ml 70% ethanol was added to rinse the silica pellets, vortexed until a suspension was formed and centrifuged at 12000 rpm for 15 seconds (no more than 20 seconds). The ethanol was removed and any remaining ethanol was taken by a micropipette. The silica pellet was re-suspended in 1 ml of ddH2O, vortexed until the silica pellet forms a suspension, incubated at 55°C for 10 minutes, homogenized with a vortex machine, and centrifuged at a speed of 12000 rpm for 2 minutes. 500 µl of the top solution was put into new microtubes and the total amplification process was performed with a PCR reaction mixture volume of 25 µL, which consists of Master Mix (KAPABiosytems, KK510) and 2 µL Forward Primer (ERB1), 2 µL Reverse Primer (ERB10), 2 µL Template DNA, and 19 µL Nuclease-free water [9,29]. The primer composition can be seen in Table 1.

Table 1. List of Primers for PCR

No	Primer	Sequence (5`-3`)	Size (bp)
1	ERB1 (F)	ACCTGGTTGATCCTGCCAG	2–20
2	ERB10 (R)	CCTCCGCAGGTTCACCTACGG	2079–2059

Source: Scientific journals [7]

Amplification

Pre-denaturation was performed at the temperature setting of 94°C for 2 minutes, followed by denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute, extension at 72°C for 1 minute 30 seconds for 35 cycles, and final elongation at 72°C for 5 minutes [29].

Electrophoresis

The results of DNA amplification were examined using agarose gel 1.5% soaked with 1X Tris Acetate-EDTA (TAE). The holes in the gel were filled sequentially with 100 bp DNA Ladder markers (Geneaid, Nexmark), 8 µL of amplification, and control blanks [29]. The PCR products were analyzed on 1.5% agarose gel containing 0.5 $mg.mL^{-1}$ ethidium bromide in 1X Tris Acetate-EDTA (TAE) buffer and the size was estimated by comparison with 100 bp of invitrogen DNA Ladder [30,31]. The electrophoresis process was carried out for 45 minutes with a voltage of 100 volts. The gel was placed in gel documentation, observed under UV light, and documented [29]. The tool for taking electrophoresis images is the BioDoc System Imaging tool.

RESULTS AND DISCUSSION

Fish Infection

Koi carp samples that were suspected to be infected with *Myxobolus koi* can be seen in Figure 1. The gills had swollen and become pale in color, and the gill cover (operculum) did not close completely (Fig. 1a). White spots also appeared on the edge of the lamella. Figure 1b shows the pale red gills along with spots on the gills. The observed primary and secondary lamellae are shown to have merged in the figure. One of the symptoms of *Myxobolus koi* infections in Koi carp is the appearance of parasite cysts in the gills [32]. The fish were in fair physical condition, though the gills were characterized grossly by widespread, multifocal coalescence, with 2–3 mm nodular white foci [9,33,34].

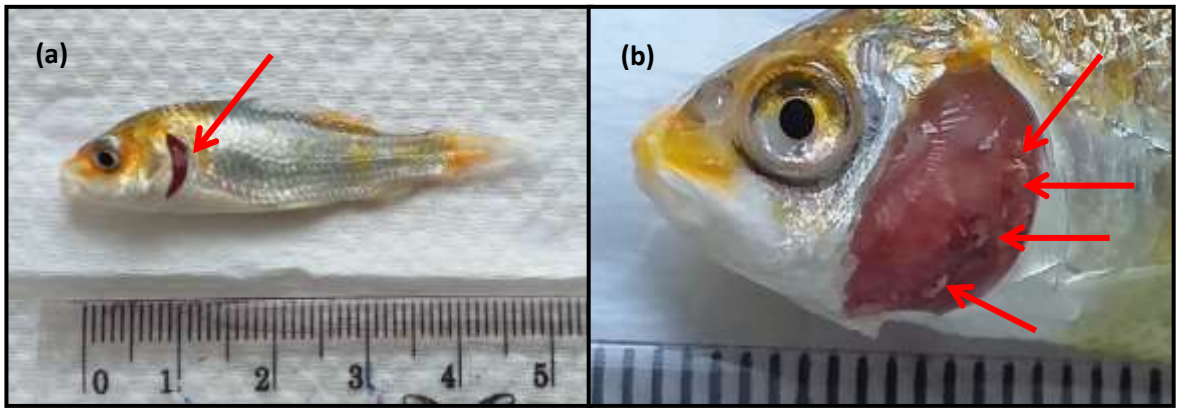

Figure 1. Koi gills infected by the *Myxobolus koi* parasite; (a) operculum did not close completely, (b) nodules in the gills

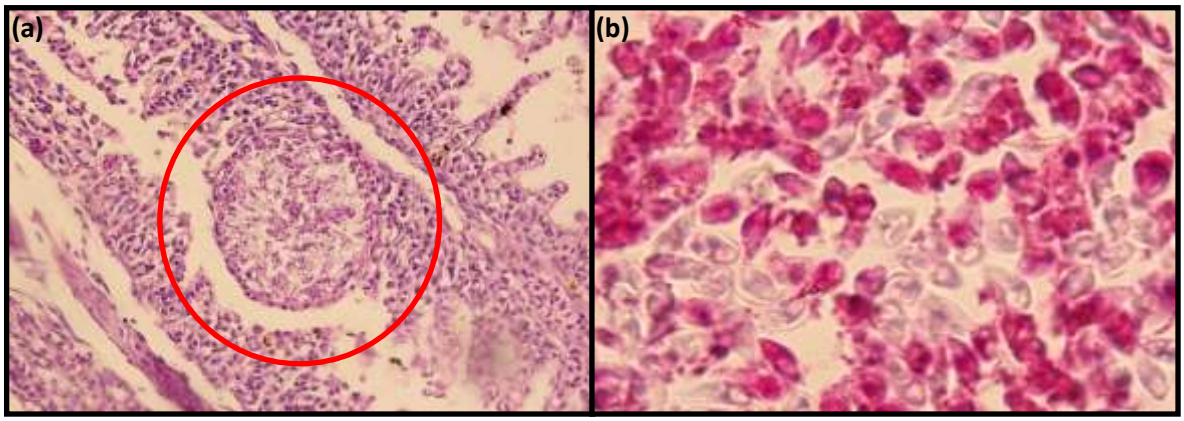

Figure 2. Histological sections of *Cyprinus carpio* gills showing location of *Myxobolus koi* tissue infection **(a)** Gills showing plasmodia in gill lamellae (100x) **(b)** *Myxobolus koi* spores in gill lamellae (1000x)

Gill Histopathology

The gills function as a breathing apparatus, but also function to regulate the exchange of salt and water between the body and the environment and play a role in removing nitrogen-containing wastes [34,35,36]. Structural damage in the gills of fish very much influences the regulation of osmosis, disrupting breathing and osmoregulation processes of fish [37,38].

Plasmodia are commonly found in gill filaments and are generally located in the middle of the gill lamella [39]. Gill histopathological studies show the number of plasmids and parasites in gills with little cell hypertrophy and epithelium [39]. Histological analysis showed that plasmodia developed in the lumen of flat capillaries and in the encapsulation process with gill filaments (Fig. 2a). *Myxobolus koi* spores in the gill lamella can be seen in Figure 2b. However, the rest of the gill lamellae and cartilage structure remained intact. There was no inflammation and swelling or infiltration of inflamed cells in one of the examined histological slides [33]. In the infected fish, there were only changes characterized by hyperplasia of the primary lamella cartilage that envelops parasitic cysts and the encapsulation process that surrounds the cysts by gill cartilage [46].

Scanning Electron Microscopy (SEM)

Some features of the myxozoan spores are more specifically disclosed through SEM. Plasmid spores that cover the gills will inhibit the process of absorption of oxygen by the gill lamella. *Myxobolus koi* specifically attacks the gills in the gill arches and filaments [32]. In Figure 3, the spores appear to break out of the gills and cover the lamella section. The spores are oval and elongated, with a size of ± 12 µm (800x).

Molecular Detection

Initially, identification with spore morphology was used for the basis of identification of myxozoan species. In the early 1990s, phenotypic features such as hosts, organs, and tissue specificity were used as important characteristics for specific identification [40,41,42]. Then,

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several studies confirmed the importance of molecular features, and in some cases, they have been found to correlate with the results of molecular data based on the 18S rDNA sequence comparison [34,43].

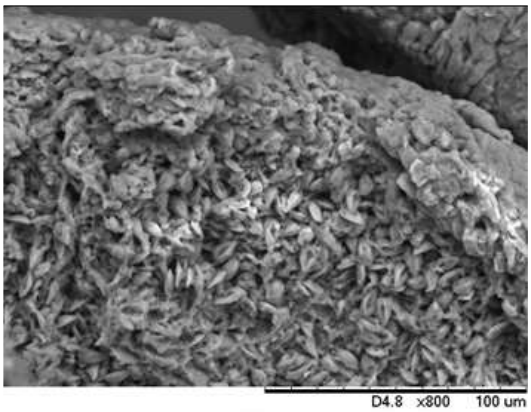

Figure 3. Scanning electron microscopy of *Myxobolus koi* spores, gill lamellae of *Cyprinus carpio* showing coverage with spores (Scale bar = 100 µm; 800x)

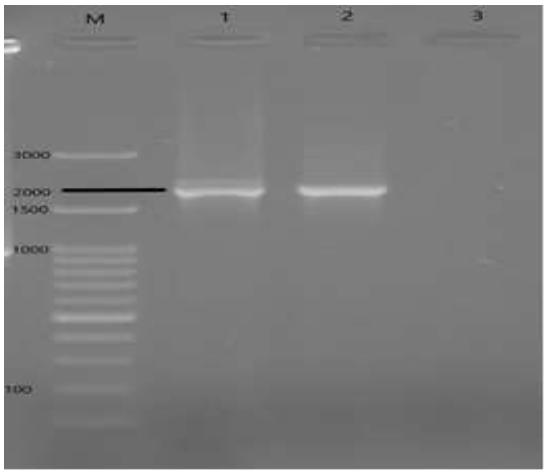

Figure 4. Agarose gel (1.5%) showing 18S r DNA gene amplification of *Myxobolus koi* DNA (2000bp) from *Cyprinus carpio*.
Description:
M: Marker/DNA ladder;
1: *Myxobolus koi* DNA (K+),
2: Positive *Myxobolus koi*,
3: Negative *Myxobolus koi*

PCR testing with specific primers in the DNA template amplification process will only display the appropriate band. DNA from gill tissue in clinically infected and uninfected fish was examined by PCR with specific primers (ERB1 Forward and ERB10 Reverse) to find the presence of the *Myxobolus koi* parasite. [44,45]. The results of electrophoresis can be detected at 2000 bp. DNA-based examination with molecular techniques has several advantages over microscopic observation because it is very specific and sensitive, and thus it can detect infections at an early stage and very mild infections [25]. In Figure 4, the appearance of the band in lane 2 shows the copy of *Myxobolus koi* genome in the target organ sample, whereas in lane 3, the band does not appear, presumably due to the absence of a copy of the *Myxobolus koi* genome or the very small volume of the genome that is undetectable with the nested PCR method.

CONCLUSION

For the present study, the myxosporean parasite *Myxobolus koi* was found to have infected the gill lamellae of *Cyprinus carpio* cultivated in Blitar, East Java. The present species was described based on morphology, SEM, histopathology, and molecular detection with specific primers. The *Myxobolus koi* parasite can be detected by positive samples marked by checking using Nested PCR, with positive control found at 2000 bp. Molecular detection with specific primers is the most effective method because it can detect early stages of infection, mild infection without clinical symptoms, and specific species that infect the host.

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Phytochemical Screening and Antibacterial Activity of Kratom Leaf (*Mitragyna speciosa* Korth.) Against *Aeromonas hydrophilla*

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Abstract

Kratom (*Mitragyna speciosa*) is an indigenous tropical herbal plant to the Northern Malay Peninsula and Thailand. Empirically kratom leaves have several properties as herbal medicines. Currently, the treatment of diseases caused by bacteria that are resistant to antibiotics requires new compounds that have high potential. The material studied was kratom leaf extract. The solvent used for extraction is methanol. Phytochemical screening carried out includes the examination of alkaloids, flavonoids, steroids/terpenoids, phenols, tannins, and saponins. The test bacteria used was *Aeromonas hydrophilla*. The medium used is Nutrient Agar (NA). NaCl 0.9% as a bacterial suspension. Testing the antibacterial activity carried out with 10 (ten) variations of concentration namely 3%, 6%, 9%, 12%, 15%, 18%, 21%, 24%, 27% and 30 %. Fresh kratom leaf is collected and then wet sorted. The extraction process was using the method of maceration. This study aims to analyze the antibacterial activity of kratom leaf, measure the secondary compounds of kratom leaf extract, and measure the best concentration to kill *Aeromonas hydrophilla.* The extraction process obtained blackish-brown extract with 62.27 grams of extract (31.14%). Secondary metabolites of kratom leaf extract show positive results of alkaloid, saponins, tannins, phenolics, steroids, and triterpenoids. Antibacterial activity against *Aeromonas hydrophila* which is characterized by a kill zone around the paper disc with the best concentration that shows a wide killing zone was 24% of the extract.

Keywords: Antibacterial, Extract, Kratom, Mitragynine, Phytochemical Screening.

INTRODUCTION

Kratom (*Mitragyna speciosa*) is a tropical herbal plant indigenous to the Northern Malay Peninsula and Thailand [1]. In Indonesia, Kratom is a typical plant of Kalimantan, especially in Putusibau, West Kalimantan. The native society knows kratom leave as *Purik* leaf [2]. Generally, kratom consumed by chewed up, smoked as cigarettes, and drink as a tea [3].

Empirically kratom leaves have several properties as herbal medicines, including as a poultice on wounds, fever medication, relieve muscle pain, reduce appetite, and treat diarrhea [1,4]. Several studies on the pharmacological effects of kratom leaf have also been investigated, such as analgesic, stimulant, antidepressant, antioxidant, anti-inflammatory, antinociceptive, and antibacterial activities [5-8]. Alkaloid extracts from *Mitragyna speciosa* leaves show antidepressant effects and can reduce the effects of alcohol dependence [9]. Kratom has been suggested as a useful constituent in the treatment of opiate addiction as a replacement therapy [10]. The leaf also reported for its antitussive, anesthetic, antinociceptive, stimulant, analgesic, and narcotic-like action properties [10-12]. However, research on antimicrobial properties of the *M. speciosa* has been few reported. In this study, we report the antimicrobial and antioxidant activities of aqueous, methanolic, and alkaloids extracts of *M. speciosa* leaf.

Currently, the treatment of diseases caused by bacteria that are resistant to antibiotics requires new compounds that have high potential. Research on antibacterial properties is required to get new antibacterial compounds that have the potential to inhibit or kill antibiotic-resistant bacteria at an affordable price. One alternative that can be taken is to utilize active bacteria-killing substances contained in plants. This study aims to analyze the antibacterial activity of kratom leaf, measure the secondary compounds of kratom leaf extract, and measure the best concentration to kill *Aeromonas hydrophilla.*

MATERIAL AND METHOD

Material

The materials used in this study are kratom leaves (*Mitragyna speciosa* Korth.) collected from Tanjung Jati Village, Putussibau Selatan District, Kapuas Hulu Regency, West Kalimantan Province in May 2019. The solvent used for extraction is methanol. The test bacteria used

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was *Aeromonas hydrophilla*. The medium used is Nutrient Agar (NA). NaCl 0.9% as a bacterial suspension. The chemicals used are hydrochloric acid, potassium hexa cyanated acid (III), iron (III) chloride, anhydrous acetic acid, chloroform, sulfuric acid, Dragendorff reagent, and Mayer reagent.

Sample Collection and Preparation

Fresh kratom leaves are collected and then wet sorted. After that, the leaves are washed and dried by shade drying in a room protected from direct sunlight. Furthermore, dried kratom leaves are sorted dry and cut into small pieces then grind till becoming *Mitragyna speciosa* Korth simplex powder.

Extraction

Simplisia powder (500 gram) was put into maceration vessels and macerated using methanol as a solvent (1:3 w/v). The extraction process using the maceration method takes approximately 5 (five) days. Aqueous extract (maceration results) then filtered using filter paper and transferred into a container. The macerate is then concentrated using a rotary evaporator and followed by evaporation on a water bath until a thick methanol extract obtained. A total of 2.08 kg of *Mitragyna speciosa* Korth simplex powder. The dried one extracted using 96% methanol solvent. Solvent replacement every 1x24 hours and macerated for 7x24 hours. Macerate is concentrated with a rotary evaporator and water bath to obtain a thick extract.

Phytochemical Screening

Phytochemical screening carried out includes examination of alkaloids, flavonoids, steroids/ terpenoids, phenols, tannins, and saponins [13].

Antibacterial Testing

Testing the antibacterial activity of kratom leaf extract is carried out by the agar diffusion method using the NA medium. The bacterial suspension of 1:40 dilution of 0.02 ml was mixed with 10 ml of NA medium in a diluent bottle, shaken out to be homogeneous, and then poured into a petri dish. Wait until the medium is solid. The paper disc was dipped in the test solution and then placed on the surface of the NA medium, which was already solid and incubated at 37°C for 24 hours. Negative control using the paper disc dipped in distilled water. Testing the antibacterial activity of kratom leaf extract against *Aeromonas hydrophila* as carried out with 10 (ten) variations of concentration namely 3%, 6%, 9%, 12%, 15%, 18%, 21%, 24%, 27% and 30 % [16].

RESULT AND DISCUSSION

Extraction Result and Secondary Metabolites

The extraction process obtained blackish-brown extract with 62.27 grams of extract and extract yield percentage of 31.14%. The results of the identification of secondary metabolites of kratom leaf methanol extract obtained positive results of alkaloid compounds, saponins, tannins, phenolics, steroids, and triterpenoids. Secondary metabolite test results can be seen in Table 1.

Table 1. Secondary metabolites content of kratom leaf extract.

Secondary Metabolic Parameter	Result
Alkaloids	+
Flavonoids	+
Saponins	+
tannins	+
phenol	+
Steroid/terpenoids	+

Based on the results of phytochemical screening stated that the kratom leaf methanol extract contains secondary metabolites of the alkaloid, flavonoid, steroid/terpenoid, phenol, tannins, and saponin groups. The results of this study support the results of previous studies, which stated that kratom leaf methanol extracts contain high concentrations of secondary metabolites of alkaloids and flavonoids, while tannins and steroids detected with low concentrations [7]. The main compounds in *Mitragyna speciosa* are alkaloids, triterpenoids, and flavonoids [14]. Besides alkaloids, kratom leaves also contain flavonoids, saponins, triterpenoids, and glycoside derivatives[15].

Antibacterial Activity of Kratom Leaf

The results of the antibacterial activity test showed that kratom leaf extract has antibacterial activity against *Aeromonas hydrophila,* which is characterized by a kill zone around the paper disc. A negative control that is distilled water does not produce a kill zone around the paper disc, so it means that distilled water has no antibacterial activity, and methanol extract of kratom leaf has antibacterial activity.

The killing zones that formed around the paper disc can be categorized base on the strength of the antibacterial activity of the extract. If the area of the resistance zone is 20 mm or more, it categorized in a powerful activity, between 10-20 mm is categorized in a strong activity, between 5-10 mm is categorized in the

moderate category and area barriers of 5 mm or less are included in the weak [16]. The measurement data of the kill zone of the methanol extract of kratom leaves can show in Table 2.

Table 2. The data of the kill zone of the extract of kratom leaves compare the negative control

Sample	Concentration (%)	Average kill zone diameter against *A. hydrophilla* (mm)
Kratom Extract	3	0
	6	0
	9	6.32
	12	6.44
	15	6.98
	18	7.32
	21	8.09
	24	9.55
	27	8.96
	30	8.23
Negative control (Distilled water)	0	0

Table 2 shows that the antibacterial activity of methanol extracts kratom leaf against *A. hydrophilla* at concentrations of 3% and 6% did not have inhibitory zones by concentrations of 9%, 12%, 15%, 18%, 21%, 27 % and 30% included in the medium category. The best concentration was at 24 %. Antibacterial activity test shows that increasing the concentration of extract given can increase the killing power of the extract against bacteria. However, if the concentration of the extract continues to increase, it can reduce the killing power. We assume that this is because an increase in concentration can lead to an increase in the viscosity of the extract, thus affecting the rate of diffusion of the extract in agar media. In addition to the concentration factor, the type of antimicrobial material can also determine the ability to inhibit bacterial growth [17]. In this study, we assumed that the antibacterial activity of kratom leaf extract was due to the presence of alkaloid compounds. Alkaloid compounds are lipophilic substances that are known to act as antimicrobials against bacteria, fungi, viruses, and protozoa by damaging bacterial cell membranes [18].

CONCLUSION

Secondary metabolites of kratom leaf methanol extract obtained positive results of alkaloid compounds, saponins, tannins, phenolics, steroids, and triterpenoids. Kratom leaf extract has antibacterial activity against *Aeromonas hydrophilla*, which is characterized by a kill zone around the paper disc. The best concentration that shows a wide killing zone was 24% of the extract.

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The Response of Botanical Seeds of *Trisula* and *Biru Lancor* Shallot Varieties to Coconut Water Treatment

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Abstract

The use of botanical seeds for seedling cultivation has problems related to the number of produced tubers, where in general botanical seeds on average only produce 1-2 tubers. Another problem with the cultivation of shallots with botanical seeds as a source of seedlings is the low growth of seeds (germination). To overcome the problems that exist in botanical seeds, growth regulators are used. This study aimed to determine the effect of coconut water treatment on the growth and yield of Trisula and Biru Lancor shallot varieties from botanical seeds, carried out from April to August 2018 in Pelem Village in Pare Sub-District, Kediri Regency. The utilized method is soaking the shallot botanical seeds for 4 hours before sowing; the seeds were then sown and planted for a month before being moved to the field. After growing in the field, observations were carried out at 1-week intervals on vegetative and generative growth. Sampling was random, and data was analyzed with t-test/LSD at a level of 5%. The results showed that the botanical seeds of Trisula and Biru Lancor shallot varieties showed different responses to the treatment with young coconut water. The use of coconut water for the Trisula variety led to 70% of plants yielding more than six cloves, while the Biru Lancor variety only had 46.67%. The use of coconut water for the Trisula variety yielded a fresh tuber weight of 71.2 g per plant at harvest; if converted per hectare, the Trisula variety can yield approximately 32 tons of fresh tubers. Meanwhile, the Biru Lancor variety yielded a tuber weight of 57.4 g per plant at harvest, and if converted per hectare, the Biru Lancor variety can yield approximately 26 tons of fresh tubers.

Keywords: Botanical seed, Coconut Water, Shallots

INTRODUCTION

Shallots are presently generally cultivated by using tubers as seedlings. There are disadvantages of using tubers as seedlings in shallot farming, one of which is that the cost of providing seed tubers is quite high, which is about 40% of the total production costs [1,2]. In addition, the health quality of seed tubers is not guaranteed because they almost always carry disease pathogens such as *Fusarium* sp. and *Colletotrichum* sp. fungi, viruses such as the Shallot Latent Virus (SLV) and Leek Yellow Stripe Virus (LYSV), as well as bacteria from their native plants which attack the plants, thus reducing yields [3,4].

The weaknesses of vegetative propagation by tubers can be sidestepped through propagation by botanical seeds of shallots. Therefore, the use of botanical seeds is one alternative that can be developed to improve the quality of shallot seeds [5-8].

The use of botanical seeds compared to the use of seed tubers (the conventional method) has several advantages. The amount of botanical seed needs is less than 3-6 kg.ha^{-1}, while the requirement of seed tubers ranges from 1-1.5 tons.ha^{-1}; botanical seeds are more accessible and cheaper in terms of storage and distribution, produce healthier plants because they are free of pathogens, and produce tubers of better quality [9,10]. In addition, the use of shallot seeds is economically feasible because it can increase yield by twofold compared to the conventional use of seed tubers [11]. Currently, the technology of producing botanical seeds of shallots has been developed, and there are relatively no problems, but the problem lies in the growth of botanical seeds. The use of botanical seeds for seedling cultivation has problems related to the number of produced tubers. Botanical seeds only produce 1-2 tubers on average [12]. The minimum number of produced tubers appears to be related to the small number of tillers produced by botanical seeds. The shallot tillers are the development of apical buds that form due to the loss of the dominance of the apical buds. The initiation of these shoots occurs after apical meristem cell division. The formation of seedlings of shallots from botanical seeds is still tricky to perform because tuber formation cannot be influenced by temperature, spacing, and water stress [13].

Another problem with the cultivation of shallots with botanical seeds as a source of

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seedlings is the low growth of seeds (germination). A growth regulator substance that is widely used in research on shallots is gibberellin (GA). The growth of shallot botanical seeds can be increased by immersion in a gibberellin solution before sowing. For three shallot varieties, the percentage of shallot seed sprouts was the best in seeds given an immersion treatment in gibberellin solution for 4 hours at the seedling age of 2 weeks [14]. The use of growth regulator substances combined with auxin, cytokinins, and gibberellins was able to increase the number of leaves, the number of tubers, and the fresh weight of plants [15]. The best and most efficient way of GA3 application for blooming, fertilizing, and yielding shallots is by soaking seed tubers for 30 minutes in a GA3 solution of 200 ppm [7].

Another substance that can be used as a growth regulator is coconut water. Coconut water not only contains vitamins and minerals [16], but also the growth hormones of auxin, cytokinins, and gibberellins in sufficient concentrations [17]. Coconut water contains the auxin hormone in the form of IAA with a level of approximately 0.237 ppm; cytokinin in the forms of kinetin with a concentration of 0.441 ppm and zeatin with a concentration of 0.247 ppm; and gibberellins in the form of GA3, GA5, and GA7 with concentrations of 0.460 ppm, 0.255 ppm, and 0.053 ppm respectively [17]. Coconut water also contains minerals that function as nutrients, which are N, P, K, Mg, Fe, Na, and Ca [16,17].

Thus, young coconut water could be used as a growth regulator in shallot cultivation by using botanical seeds as a seed source. Treatment of coconut water on shallot botanical seeds of the Trisula variety and sowing on soil mixture + manure, soil mixture + husk charcoal, and soil mixture + compost + husk charcoal can increase sprout growth by 90% and optimize seed growth [18]. Shallot plants originating from botanical seeds that are treated with coconut water, in addition to having optimum plant growth, also yielded the highest number of tubers per plant and the highest tuber weights per plant [19]. The use of coconut water for the treatment of shallot botanical seeds will make it easier for farmers, considering that coconut water is easily obtained and inexpensive.

The aim of this study is to determine the effect of coconut water treatment on the growth and yield of *Trisula* and *Biru Lancor* shallot varieties grown from botanical seeds.

METHODOLOGY

The study was conducted at Pelem Village in Pare District, Kediri Regency, from April to August 2019. The study was performed using a randomized block design that was repeated ten times. Botanical seed nursery was carried out from April-May 2018. Before the seeds were sown, they were first soaked in a coconut water solution for 4 hours. This soaking process was for the botanical seeds to absorb the compounds in coconut water. Seed germination is a mechanism in which morphological and physiological alterations result in activation of the embryo. Before germination, seeds absorb water, resulting in the expansion and elongation of the seed embryo [20]. Next, the seeds were planted in a plastic bag containing a mixture of soil and compost media with a ratio of 1:1. The seeds were sown until the age of 1 month after seedling growth, and then the shallot seedlings were planted in the field.

Each shallot variety (*Trisula* and *Biru Lancor*) was planted in 10 beds measuring 5 m x 4 m. Shallot seeds were planted on beds with a spacing of 12.5 cm x 15 cm. Total of 10 plants were selected randomly as samples for observation of plant growth. Observation of vegetative growth (number of leaves, plant height) and generative growth (number of stems/tillers) was carried out at one-week intervals, while the observation of yield components (number of tubers, tuber weight per plant) was carried out at harvest. The variable analysis was carried out to determine the effect of coconut water treatment and continued with t-test/LSD at a level of 5%.

Figure 1. Shallot Botanical Seeds

RESULTS AND DISCUSSION

Vegetative Growth

The results of statistical analysis showed that the treatment of coconut water affected the vegetative growth of the Trisula and Biru Lancor varieties. The Trisula variety showed better

growth in plant height, number of leaves, and number of stems compared to the Biru Lancor variety. The performance of vegetative growth (plant height, number of leaves, and number of stems) of the Trisula and Biru Lancor varieties are presented in Tables 1, 2, and 3, respectively.

Table 1. Plant height of the *Trisula* and *Biru Lancor* shallot varieties after transplanting

Variety	Plant Height (cm)			
	2 WAT	3 WAT	4 WAT	5 WAT
Trisula	32.18 a	37.22 a	46.03 a	51.82 a
Biru Lancor	28.73 b	34.42 b	41.28 b	47.53 b

Notes: Numbers in the same column followed by different letters are significantly different based on the t-test with a level of 5%. WAT: Weeks after Transplanting

Table 2. Number of leaves of *Trisula* and *Biru Lancor* shallot varieties after transplanting

Variety	Number of Leaves			
	2 WAT	3 WAT	4 WAT	5 WAT
Trisula	12.53 a	17.27 a	22.13 a	25.07 a
Biru Lancor	14.50 b	15.30 b	22.67 a	29.33 b

Notes: Numbers in the same column followed by different letters are significantly different based on the t-test with a level of 5%. WAT: Weeks after Transplanting

Table 3. Number of branches of the *Trisula* and *Biru Lancor* shallot varieties after transplanting

Variety	Number of Branches			
	2 WAT	3 WAT	4 WAT	5 WAT
Trisula	3.13 a	4.33 a	5.53 a	6.27 a
Biru Lancor	2.90 a	3.17 b	4.47 b	5.87 a

Notes: Numbers in the same column followed by different letters are significantly different based on the t-test with a level of 5%.
WAT: Weeks after Transplanting

Based on Tables 3, 4, and 5, the treatment of coconut water immersion resulted in good/optimal vegetative growth, although there were differences in vegetative growth between the Trisula and Biru Lancor varieties. Another research had shown that coconut water immersion influenced the growth of mustard plants, mainly on growth components of plant height and number of leaves [21]. In addition, another study had also shown that the application of coconut water increased the growth of *in vitro* ginger [22]. The coconut water is an endosperm liquid containing organic compounds, including auxin and cytokinin [23]. When immersed in a young coconut solution, botanical seed tissues absorb auxin and cytokinin, thereby increasing cell division, elongation, and differentiation of cells, which ultimately increases plant growth.

Plant growth is influenced by the availability and distribution of food in other parts of plants, the uptake of water or nutrients by parts of plants, the presence of growth regulators, or the formation of certain substances in plants [24]. Furthermore, the presence of auxin in plants not only spurs stem lengthening but also encourages the growth of all parts of plants, including roots and leaves [25]. In addition to containing the growth hormones of auxin, cytokinin, and gibberellin, coconut water also includes the nitrogen (N) nutrient. Nitrogen is needed by plants to encourage vegetative growth. Plants require nitrogen (N) for vegetative growth of leaves, stems, and roots [26].

Components of Generative Growth and Results

At the age of 70 days after transplanting, the shallot plants are ready to be harvested, which is marked by 60% soft neck stems, yellow leaves, and fallen plants (Fig. 2). Statistical analysis showed that the treatment of immersion in coconut water for botanical seeds influenced the generative growth of shallots, including the number of tillers/stems, number of tubers per plant, tuber weight per plant, and yield/production.

Figure 2. Shallot plants from botanical seeds that are ready to be harvested

At harvest, the *Trisula* shallot variety from botanical seeds showed greater generative performance (number of branches), yield components (tuber number per plant, tuber weight per plant), and yields, compared to the Biru Lancor variety from botanical seeds (Table 4).

The components of the Trisula variety of shallots from botanical seeds are better than the Biru Lancor variety, where the tuber weight per plant is greater. The greater tuber weight per plant seems to be related to the number of branches/tillers and the number of tubers per

plant. Based on Table 4, there appears to be a correlation of the number of branches/tillers, the number of tubers per plant, and tuber weight per plant toward production.

Table 4. Generative and yield components of the *Trisula* and *Biru Lancor* shallot varieties from botanical seeds

Variety	Generative and Yield Components			
	Number of Branches	Number of Tubers per plant	Tuber Weight per plant (g)	Production (tons.ha^{-1})
Trisula	6.8 a	7.3 a	71.2 a	32.0 a
Biru Lancor	6.0 a	6.5 a	57.4 b	26.0 b

Notes: Numbers in the same column followed by different letters are significantly different based on the t-test with a level of 5%.

The Trisula variety produces a greater number of branches/tillers, number of tubers per plant, and tuber weight per plant, and has higher production than the Biru Lancor variety. The Biru Lancor variety had a production of 26 tons.ha^{-1}, less than the Trisula variety. The Trisula variety showed more branches/tillers and tubers per plant than the Biru Lancor variety (Fig. 3). Although the number of tubers per plant was lower than the Trisula variety, the Biru Lancor variety showed an average number of 6 tubers per plant. This fact indicates that coconut water can be used as a solution to the problem of low number of tubers produced by shallot plants from botanical seeds.

When examined further, 70% of the Trisula variety of shallot plants from botanical seeds produced more than 6 tubers per plant, while for the Biru Lancor variety, only 46.67% produced the same number of tubers. In detail, the percentages of plants with 4, 5, 6, and more than 6 tubers, for both the Trisula and Biru Lancor varieties, are presented in Table 5.

Table 5 shows that the treatment of coconut water can increase the number of shallot tubers. The results of this study are in line with Rajiman [27] who reported that the provision of real coconut water affects the vegetative growth of shallot plants, especially the number of leaves, as well as their generative growth, namely the number of tubers, tuber diameter, and fresh and dry weight per clump. The provision of coconut water affected the number of shallot tubers [28]. Coconut water could increase the growth and production of shallots [29].

Figure 3. Performance of Trisula (TS) and Biru Lancor (BL) shallot varieties from botanical seeds

Table 5. Percentages of plants with 4, 5, 6, and more than 6 tubers of Trisula and Biru Lancor shallot varieties from botanical seeds

Variety	Percentage of tubers per plant (%)			
	4 tubers	5 tubers	6 tubers	>6 tubers
Trisula	6.67 a	10.00 a	13.33 a	70.00 a
Biru Lancor	13.33 b	20.00 b	20.00 b	46.67 b

Notes: Numbers in the same column followed by different letters are significantly different based on the t-test with a level of 5%.

CONCLUSION

Botanical seeds of the Trisula and Biru Lancor shallot varieties showed different responses to the treatment with young coconut water. The treatment of coconut water for the Trisula variety enabled 70% of plants to be capable of producing of 6 or more tubers, while for the Biru Lancor variety only enabled 46.67%. Coconut water treatment for the Trisula variety can yield 71.2 g of fresh tuber weight per plant at harvest time, and if converted per hectare, the Trisula variety can yield up to 32 tons of fresh tubers. Meanwhile, the Biru Lancor variety can yield 57.4 g of fresh tuber weight per plant at harvest time; if converted per hectare, the Biru Lancor variety can yield up to 26 tons of fresh tubers.

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Effect of Five Types Inert Dust to *Rhyzopertha dominica* (Fabricius) (Coleoptera: Bostrichidae) in Stored Rice Seeds

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Abstract

Rhyzopertha dominica (F.) is one of the primary pests in stored rice. Generally, pest control in the storage was done by spraying and fumigation using synthetic insecticide. The application of chemical insecticide using phosphine can cause resistant to insect pests and toxic to humans. Regarding the negative effect of insecticide application, the alternative control by using natural resources like inert dust is expected to be a good solution to control pests of stored products. This research was aimed to study the effectiveness of five inert dusts, which are: rice husks ash, volcanic ash, giant bamboo leaves ash, corncobs ash, coconut shells ash to adult mortality and the inhibition of population growth of *R. dominica* on stored rice seeds. The results showed that giant bamboo leaves at 8 g kg^{-1} caused 100% of mortality and faster than other inert dusts tested. Giant bamboo leaves and rice husks were more effective to suppress the number of eggs, larvae, pupae, and new adults (F1). Inert dust could decrease the hatchability of eggs, inhibit population growth, and decrease the weight of new adults (F1). The damage to the treated rice seeds was significantly lower than untreated rice seeds.

Keywords: ash, control, inert dust, silica, stored product pest

INTRODUCTION

Lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae) is the primary pest in stored rice, tobacco, beans, cacao bean, biscuits, and some dried fruits [1,2]. Infestation of pest stored product can cause losses from 5-10% of total global production, while the losses in tropical and subtropical countries could reach up to 50% [3]. The destructive phase is on larvae and adult. Larvae feed on the embryonic and endosperm of seed, and it cause the un-germinated seed, while adult feed on the surface layer of the seed irregularly [4].

Pest control in the storage nowadays is based on insecticide by spraying and fumigation using carbon monoxide gas (CO). Fumigation technique can only applied on the sealed storage [5]. Various types of insecticides which occasionally used in the storage are organophosphate and carbamates for spraying, while phosphine for fumigation [6,7]. Misuse of organophosphate can cause poisoning and death [8-11]. Furthermore, using phosphine at high dose and unwise application can cause pest resistant.

Inert dust application is one of the alternative control that can be easily adopted by farmers. Inert dust application as grain protectant has been used since the Aztecs of ancient Mexico which used mixed lime and corn to protect their seeds. However, this materials could not be commercialize anymore in the last fifty years [12]. The mechanisms of inert dust to insect body is by removing the body fluid of insect and eroding the integument, this condition cause an excessive evaporation on its body and eventually cause death [13]. Silica (SiO_2) content in inert dust material can cause insect death because of the fat layer on cuticles is absorbed by silica reaction. Several materials that have high silica content and easy to find in Indonesia are volcanic ash (45.70%), rice husks (79%), giant bamboo leaves (58.30%), corncobs (47.78%), and coconut shells (45.05%) [14-18]. This research was aimed to study the effectiveness of five types of ash as an inert dust materials i.e. volcanic ash, rice husks, giant bamboo leaves, corncobs, and coconut shells to adult mortality and the inhibition of population growth of *R. dominica* on stored rice seeds.

METHOD

The research was conducted at Plant Pest Laboratory, Department of Plant Pests and Diseases, Faculty of Agriculture, University of Brawijaya.

Insect Diet

Brown rice Ciherang variety was used for *R. dominica* rearing and White rice seed Ciherang variety was used in the experiment. These materials were removed from debris i.e. rocks, husks, damage seeds, and insect infestation. Cold

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sterilization process was done for both materials [19,20]. These materials was put into a glass tube (Ø= 13 cm; h= 25 cm) during the sterilization process in the freezer at -15°C for seven days then removed to refrigerator at 5°C for seven days and removed to the laboratory room (27±2°C; 65% RH) for fourteen days.

Insect Rearing

The *R. dominica* was obtained from Assessment Institute for Agricultural Technology, Karangploso, Malang. Insect rearing was done by infest 200 adults insect into 500 g of brown rice for seven days in the glass tube (Ø= 14.5 cm; h= 16 cm) and covered by gauze materials for air circulation [19]. After seven days infestation, adult infestation were removed and the feed was incubated until F1 progeny emerged as the treated insect. Adults sexes was distinguish by the colour of abdominal segments [19].

Inert Dust Preparation

Five types of ash as inert dust materials that used in this research were from Bromo mountain volcanic ash, rice husks, corncobs, giant bamboo leaves, and coconut shells. All materials were dried and burned separately. Ashes were sieved using 53 µm and dried in the oven at 60°C for one hour to reduce the water content [21]. Two levels of dose were used in this research, i.e. 4 and 8 g kg^{-1} of rice seed.

Inert Dust Content Analysis

This analysis was carried out in Central Laboratory, Faculty of Mathematics and Natural Sciences, State University of Malang. Analysis of silica level was done by X-ray Fluorescent (XRF) technique to determine the content of chemical composition on five type's inert dust, i.e. rice husks ash, volcanic ash, giant bamboo leaves ash, corncobs ash, and coconut shells ash.

Adult Mortality

Glass tube (Ø= 6 cm; h= 12 cm) contained of 100 g of rice seeds was mixed by each dose of five types inert dust regarding the treatment. Rice seeds and inert dust were mixed manually for two minutes [22]. 25 paired of adults (7–14 days old) were infested to the glass tube. Adults mortality was observed on 1, 3, 7, and 10 days after infestation (DAI) [23] and calculated by this formula [24]:

$$\text{Adult mortality (\%)} = \frac{\text{Number of dead adults}}{\text{Number of adults infestation}} \times 100\%$$

If there were dead adults in the control, the adult mortality was corrected using this formula [25]:

$$\text{Corrected mortality (\%)} = \left(\frac{X-Y}{X}\right) 100\ \%$$

Where, X: number of live adults in control
Y: number of live adults in treatment

Insect Population Growth

Total of 600 rice seeds mixed with each inert dust types and two dose levels regarding the treatment in each glass tube (Ø= 4 cm; h= 6.5 cm). Number of eggs, larvae, and pupae were calculated on 10, 29, and 35 DAI. The eggs were observed by looking the presence of oviposition site on the rice seeds surface. Larvae and pupae were observed by destructive method, while adults were observed daily from the first day of progeny emerged (F1) until all F1 emerged before F2 generation appeared [26,27]. F1 weight was also calculated as the observed variables.

Seed Damage

The seed damage was determined by seed germination test without cleaning the inert dust. 100 seeds was randomly sampled and germinated on plastic trays (33 x 23 cm). The seeds were observed after 7 days germinated [28]. Un-germinated seeds in 7 days were classified as damaged seed. The percentage of seed damage was calculated by this formula [29]:

$$\text{Seed damage (\%)} = \frac{\text{Number of damaged seeds}}{100} \times 100\ \%$$

RESULTS AND DISCUSSION

Adult mortality

Application of five types inert dust on rice seeds was significantly affect the adult mortality of *R. dominica* on 1 DAI ($F_{10,32}$=159.04; P<0.0001), 3 DAI ($F_{10,32}$= 69.001; P<0.0001), 7 DAI ($F_{10,32}$= 524.974; P<0.0001), and 10 DAI ($F_{10,32}$ = 844.786; P<0.0001). Adult mortality on giant bamboo leaves ash at 8 g kg^{-1} dose was significantly higher than other treatments (99.33%) on 1 DAI. Giant bamboo leaves ash at 4 and 8 g kg^{-1} caused total death of adults on 3 DAI and was significantly higher of adult mortality than other treatments. As well as on rice husk ash on 7 DAI at both doses caused total death of adults and was significantly different with other treatments (Table 1).

The death of insect was happened because when the insect body passed through the grain layer, inert dust particles attached on the insect cuticle. Silica content on inert dust would react with insect body and caused the desiccation on wax layer of the cuticle, thus water content loss from their bodies [30]. According to analysis of silica content (SiO_2) by XRF method was relatively higher in rice husks ash (77.7%) and giant

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bamboo leaves (51%) compared to volcanic ash (39.3%), corncobs ash (29.3%) and coconut shells ash (25%). Correlation analysis results revealed that silica content was positively correlated with adult mortality (r = 0.788; P = 0.007). Longer duration exposure of inert dust could increase adult mortality. This was in line with the previous research on *Sitophilus zeamais* Motsch. (Coleoptera: Curculionidae) which showed that adult mortality was increase followed by the exposure time of inert dust application [31]. Athanassiou *et al*. also stated that the exposure time was crucial for effective application of diatomaceous earth materials because it was related to the insect movement [32].

Insect Population Growth

Application of five types inert dust on rice seed was significantly reduce the insect population growth of *R. dominica* on different life stages, i.e. number of eggs ($F_{10,32}$=34.36; P<0.0001), larvae ($F_{10,32}$= 36.48; P<0.0001), pupae ($F_{10,32}$ =37.43; P<0.0001), and new adults ($F_{10,32}$ = 36.64; P<0.0001). The number of eggs, larvae, pupae, and new adults in giant bamboo leaves ash at both doses was significantly lower than other treatments and was not significantly different with rice husks ash at both doses. The new adults were not emerged on the giant bamboo leaves ash and rice husks ash treatment at both doses (Table 2).

Number of eggs on giant bamboo leaves ash and rice husks ash were lower because there had higher mortality than other treatments. The speed of inert dust causing adult mortality was affected on the number of eggs laid. The faster of adult mortality, the more of eggs laid [33]. Eggs hatchability to larvae on treated seeds were lower than control because the eggs which laid on the outer surface of grain was directly contact with inert dust. Previous research also mentioned that eggs hatchability of *Tribolium castaneum* (Herbst), *Tenebroides mauritanicus* L. (Coleoptera: Tenebrionidae), *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae), *R. dominica* and *Callosobruchus chinensis* (L.) (Coleoptera: Chrysomelidae) was lower when the eggs were exposed by diatomaceous earth [33]. The most effective period to apply inert dust was on the stages which directly exposed by inert dust, particularly on newly hatched larvae [33]. Moreover, application of diatomaceous earth can reduce the insect offspring by increasing adults mortality and decreasing oviposition [34].

Table 1. Adult mortality on treated rice seeds by five types of inert dust

Treatment (Ash)	Adult mortality (%)*			
	1 DAI	3 DAI	7 DAI	10 DAI
Untreated (Control)	0.00±0.00 a	0.00±0.00 a	0.00±0.00 a	0.00±0.00 a
Rice husks 4 g kg^{-1}	71.33±5.03 c	88.00±5.29 e	100.0±0.00 e	100.0±0.00 f
Rice husks 8 g kg^{-1}	74.00±6.00 c	90.00±5.29 e	100.0±0.00 e	100.0±0.00 f
Volcanic 4 g kg^{-1}	2.00±2.00 ab	10.00±5.29 b	47.67±4.06 b	62.67±5.05 b
Volcanic 8 g kg^{-1}	4.00±4.00 b	18.00±12.35 bc	59.67±0.71 c	67.00±3.00 b
Giant bamboo leaves 4 g kg^{-1}	88.00±5.29 c	100.0±3.08 f	100.0±0.00 e	100.0±0.00 f
Giant bamboo leaves 8 g kg^{-1}	93.33±4.64 d	100.0±3.46 f	100.0±0.00 e	100.0±0.00 f
Corncobs 4 g kg^{-1}	2.00±2.00 ab	21.33±11.73 bc	63.67±4.95 cd	79.00±1.73 de
Corncobs 8 g kg^{-1}	3.33±4.16 b	21.33±16.17 bc	68.67±7.38 d	72.33±3.00 c
Coconut shells 4 g kg^{-1}	0.00±0.00 a	24.67±11.55 cd	61.67±4.74 c	80.33±4.06 e
Coconut shells 8 g kg^{-1}	2.00±2.31 b	40.00±16.00 d	61.67±3.54 c	79.00±2.65 de

Notes: * Mean ± SD followed by different letters in the same column are significantly different by Duncan's Multiple Range Test (α<0.05). Data are transformed by Arcsin. DAI: day after infestation.

Table 2. Number of eggs, larvae, pupae, and new adults on treated rice seeds by five types of inert dust

Treatment	No. of eggs*	No. of larvae*	No. of pupae*	No. of new adults*
Untreated (Control)	142.00±20.46 d	122.22±34.65 c	117.78±30.96 d	91.11±17.03 d
Rice husks 4 g kg^{-1}	26.67± 6.50 a	13.33± 6.50 a	4.44± 3.69 a	0.00± 0.00 a
Rice husks 8 g kg^{-1}	20.00± 7.00 a	4.44± 6.85 a	2.22± 3.69 a	0.00± 0.00 a
Volcanic 4 g kg^{-1}	71.11±10.15 c	51.11±10.15 b	40.00± 7.00 c	33.33± 6.51 c
Volcanic 8 g kg^{-1}	60.00± 7.00 bc	33.33±07.00 b	33.33±11.55 bc	26.67±13.50 bc
Giant bamboo leaves 4 g kg^{-1}	20.00± 7.00 a	0.00± 0.00 a	0.00± 0.00 a	0.00± 0.00 a
Giant bamboo leaves 8 g kg^{-1}	15.56± 4.04 a	0.00± 0.00 a	0.00± 0.00 a	0.00± 0.00 a
Corncobs 4 g kg^{-1}	48.89±10.15 b	35.56±10.26 b	26.67± 6.51 b	17.78± 8.08 b
Corncobs 8 g kg^{-1}	55.55±10.26 bc	37.78± 6.51 b	28.89± 3.46 b	20.00± 7.00 bc
Coconut shells 4 g kg^{-1}	62.22± 4.04 bc	33.33± 6.51 b	26.67± 6.51 b	20.00± 7.00 bc
Coconut shells 8 g kg^{-1}	62.22± 8.08 bc	40.00±13.00 b	31.11±10.15 bc	22.22± 8.08 bc

Notes: *Mean ± SD values followed by different letters in the same column are significantly different by Duncan's Multiple Range Test (α < 0.05). Data are transformed by square root.

Table 3. New adults (F1) weight on treated rice seeds by five types of inert dust

Treatment	New adults weight (mg/adult)*	
Untreated (Control)	1.23 ± 0.07	D
Rice husks 4 g kg^{-1}	-	
Rice husks 8 g kg^{-1}	-	
Volcanic 4 g kg^{-1}	1.12 ± 0.06	abc
Volcanic 8 g kg^{-1}	1.06 ± 0.02	C
Giant bamboo leaves 4 g kg^{-1}	-	
Giant bamboo leaves 8 g kg^{-1}	-	
Corncobs 4 g kg^{-1}	1.06 ± 0.05	abc
Corncobs 8 g kg^{-1}	1.02 ± 0.09	A
Coconut shells 4 g kg^{-1}	1.04 ± 0.10	Ab
Coconut shells 8 g kg^{-1}	1.09 ± 0.07	Bc

Notes: *Means ± SD followed by different letters in the same column are significantly different by Duncan's Multiple Range Test ($\alpha < 0.05$). Data are transformed by square root.

The weight of new adults (F1) on treated seeds (volcanic ash, corncobs ash, coconut shells ash) were significantly reduce than control ($F_{6,20} = 15.31$; $P<0.0001$) (Table 3). This was happened because of the silica content was able to reduce the cuticle function of insect body. Rice seeds were harder due to inert dust mechanism by adsorbing the water content of seed. Therefore, insect feeding activity was disrupted and caused smaller and lighter on F1 weight.

Hygroscopic silica was able to reduce the moisture content of insect body which cause weight decreased due to the fluid loss from its body [35]. Alexander *et al*. also explained that inert dust application can reduce 30% of *Sitophilus granarius* L. adult weight [36]. Moreover, the desiccation of insect body cause water loss and the insect will be died if they lose 60% of body fluids or 30% of body weight [37].

Seed Damage

Application of five types inert dust on rice seeds was significantly reduce the percentage of seed damage by *R. dominica* than control ($F_{10,32} = 35.39$; $P<0.0001$). The percentage of seed damage on giant bamboo leaves ash at 8 g kg^{-1} was not significantly different at 4 g kg^{-1}, but it was significantly lower than other treatments (Table 4). The percentage of seed damage was influenced by the mortality of infested adults and the population growth of *R. dominica* on each treatment. The lower seed damage on giant bamboo leaves ash was caused by the high mortality of infested adults and lower hatched larvae, thus their feeding activity was lower than other treatments.

The viability of maize seed by *S. zeamais* was influenced by the number of larvae and adults which are actively damaged the stored products [31]. Larvae and adults feed on the seed embryo and endosperm, therefore the seeds unable to germinate. Newly hatched larvae was feed on the flour dust due to the results of adults attacked, while a certain larvae instar could enter the seed by boring the grain surface [7]. This holes causes the seed damage and reduce the germination potential or even un-germinated. Furthermore, it was revealed that the application of rice husk ash, tomaren indispron-P406, and cow feces to the seed which attacked by *Sitotroga cerealella* in the storage were able to keep the seed germination up to 90% for 3 months and 84.67% for 6 months [38].

Table 4. The percentage of seed damage on treated rice seeds by five types of inert dust

Treatment	Seed damage (%)*	
Untreated (Control)	29.00 ± 1.00	h
Rice husks 4 g kg^{-1}	12.33 ± 1.27	cd
Rice husks 8 g kg^{-1}	11.00 ± 1.00	bc
Volcanic 4 g kg^{-1}	19.67 ± 1.53	g
Volcanic 8 g kg^{-1}	15.67 ± 2.08	ef
Giant bamboo leaves 4 g kg^{-1}	8.67 ± 1.15	ab
Giant bamboo leaves 8 g kg^{-1}	6.67 ± 3.06	a
Corncobs 4 g kg^{-1}	18.00 ± 1.00	fg
Corncobs 8 g kg^{-1}	14.33 ± 1.15	de
Coconut shells 4 g kg^{-1}	18.00 ± 2.00	fg
Coconut shells 8 g kg^{-1}	15.33 ± 2.00	def

Notes: * Mean ± SD followed by different letters in the same column are significantly different by Duncan's Multiple Range Test ($\alpha < 0.05$).

CONCLUSION

The application of giant bamboo leaves ash and rice husks ash as inert dust at 4 and 8 g kg^{-1} was more effective to increase the adult mortality, inhibit insect population growth, and decrease the seed damage compared to volcanic, corncobs, and coconut shells ash at the same dose in stored rice seeds. In conclusion, these ashes were potentially used as a grain protectant against *R. dominica* in stored rice seeds.

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The Effect of *Moringa oleifera* Leaves and VipAlbumin® on The Immune System of Diabetes Mellitus Balb/C Mice Model

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Abstract

Diabetes Mellitus is known as a disease grown worldwide rapidly prevalent day by day. This disease caused by a chronic hyperglycemic condition and also glucose intolerance resulting from defects in insulin secretion, insulin action, or both. *Moringa oleifera* is one of the plants used in most developing countries for traditional medicine for treating diabetes. In this research, *Moringa oleifera* mixed with VipAlbumin®, taken from snakehead fish albumin. Albumin found as an antioxidant against ROS. This research aimed to evaluate the effect of *Moringa oleifera* mixed with VipAlbumin® supplement towards the profile changing of T-cell, (CD4+ CD8+) B220+, CD4+ IFN-γ, and TNF-α. The experiments were done by dividing Balb/c mice into five groups and induced with 100 mg.BW^{-1} STZ. Afterward, the mix of Moringa oleifera and VipAlbumin® orally administrated into five different doses. Negative control contains of healthy mice, Positive dose administrated with 145 mg.kg bw^{-1} STZ, Dose 1 of 100 mg.kg bw^{-1} (Mo) + 416.25 mg.kg bw^{-1} (A), Dose 2 of 150 mg.kg bw^{-1} (Mo) + 208.15 mg.kg bw^{-1} (A), Dose 3 of 50 mg.kg bw^{-1} (Mo) + 624.375 mg.kg bw^{-1} (A). One way ANOVA was applied to analyze the data with p-value 0.05% and combined with Tukey test using SPSS version 16 for Windows. The results showed that a relative number of CD4 and CD8 T cells decreased in dose 3 of *Moringa oleifera* and Albumin, as well as B220 in dose 3 gave a significant decreased compared to healthy mice ($p<0.05$). The inflammation showed decreasing after treatment with dose 3 of *Moringa oleifera* and albumin extract. Taken together that proinflammatory cytokines decreased after treatment compared to a positive group.

Keywords: Albumin, Inflammation, *Moringa oleifera*, T cells.

INTRODUCTION

Diabetes Mellitus (DM) is one of the autoimmune disease caused by the destruction and secretion of insulin production in pancreatic β-cells [1]. There are two types of DM. Insulin Dependent DM (IDDM) is known caused by a lack of insulin secretion by β-cells of the pancreas. While on the other hand, Non-Insulin Dependent DM (NIDDM) caused by decreased sensitivity to insulin of target tissues or cells, which often called insulin resistance (IR).

Inflammation was first linked to insulin resistance and diabetes in the early 1990s and could increase TNF-α in adipose tissue [2]. Inflammatory cytokines such as IL-1β and IFN-γ, which increased in obesity, also modulate insulin signaling. Furthermore, previous research asserts macrophages are the primary inflammatory cell type in the glucose utilizing tissues such as adipose tissue and liver [3].

Obesity and systemic inflammation are known to induce insulin resistance. The sign could be seen mostly in the adipose tissue, which releases fatty acids, adipokines, and other cytokines with the capability to downstream effects on the muscle and liver [4].

Another feature of insulin resistance is an increased release of free fatty acid (FFA) [5]. Obesity, characterized as a state of chronic low-grade inflammation caused by overnutrition, is a major cause of decreased insulin sensitivity, which makes obesity a major risk factor for IR.

Tumor Necroses Factor-α (TNF-α) reported by as a proinflammatory cytokine that induces insulin resistance [4,6]. Proinflammatory molecules such as leptin, monocyte, resistin, visfatin, interleukin-6 (IL-6), chemoattractant protein-1 (MCP-1), and (TNF-α), are expressed at high levels in macrophages and other cells when activated [7].

Nowadays, it is estimated that more than 80% of people in developing countries use medicinal herbs to maintain their health [8]. They use of plants in the diabetic management provides better alternatives as they are less toxic, readily available, and affordable. Since there is the fact that oxidant stress involved in diabetes, this research used herbals, which comprise anti-diabetic and also antioxidant.

One of the plants used for traditional medicine for treating diabetes in developing countries is *Moringa oleifera.* The efficacy of *Moringa oleifera* has been reported by many researchers. The root of *M. oleifera* was able to reduce stone forming constituents in the kidneys and also reduce the elevated urinary oxalate of calculogenic rats as a result of ethylene glycol treatment [9]. The positive effect of ethanolic leaf extract to insulin resistance and beta-cell

function in hyperglycemia HFD/STZ which induced to diabetic mice has also reported [10].

Reactive Oxygen Species (ROS) will increase in the individual suffering from hyperglycemia and lead to oxidative stress [11]. However, an exogenous antioxidant from *M. oleifera* can overcome oxidative stress. Another strong antioxidant can be obtained from snakehead fish *(Channa striata*) [11]. This fish albumin found to act as an antioxidant and also will overflow group (-SH), which serves as a binder radical that plays a role in the arrest of ROS.

This research used VipAlbumin® as a supplement from snakehead fish *(Ophiocephalus stiatus)* with a high content of albumin compared to the other kinds of fish. One of the albumin's benefits is as antiinflammation and antioxidant. Therefore, this study conducted to determine the effect of VipAlbumin® in change quantitative T cell lymphocytes and a decrease in blood glucose levels [12]. This research was aimed to analyze the influence *M. oleifera* and Albumin extract towards the pro-inflammation cytokine such as TNF-α, IFN-γ, and IL-6 in diabetics Balb/c mice.

MATERIALS AND METHODS

STZ Induction

Female Balb/c mice (n=25, 6-7 weeks old, body weight ±25-30 g) were acclimatized for seven days and fed with standard feed *ad libitum*. The mice assigned into five groups. There are negative control, positive DM, Dose 1, Dose 2, and Dose 3. Each group will have five times of repeated treatment. The mice in the DM group and D1, D2, and D3 intraperitoneally injected with STZ at a dose of 145 $mg.kg^{-1}$ of body weight. Afterward, mice were kept for 14 days. In the incubation period, blood glucose was checked every three days after STZ induced. Mice were reported to suffer from type 2 diabetes when its blood glucose levels exceed 200 $mg.dL^{-1}$.

Oral Administration of *M. oleifera* and VipAlbumin®

Moringa oleifera samples taken from leaves (100 g) were dried and powdered using a blender then mix with 100 mL of distilled water for 24 hours and then stored at 4°C. Afterward, the mixture was filtered twice through a 2-µm pore filter paper, then stored at 4°C for five days. Albumin extract using VipAlbumin®, which contains 500 mg of Snakehead fish extract and 30,20% albumin, and also vitamin A, vitamin D, and calcium.

The female balb/c mice divided into five different groups following the oral administration of *M. oleifera* and VipAlbumin®. The five groups are as follows (Table 1).

Table 1. Five Groups of Balb/c Mice

Group	($mg.kg^{-1}$ Body Weight)		
	STZ injection	*M. oleifera* extract	VipAlbumin®
Normal	-	-	-
DM	145	-	-
Dose 1 (D1)	145	100	416.25
Dose 2 (D2)	145	150	208.15
Dose 3 (D3)	145	50	624.375

Notes: Normal = healthy mice (negative control); DM= mice with diabetes mellitus by STZ Injection (positive control).

Spleen Cells Isolation

The mice were killed by dislocation in their neck. The spleen was separated from the mice's body and washed with sterile PBS solution and laying down on the sterile Petri dish containing 2 mL of sterile PBS. Afterward, the puree spleens were taken with the spuit in one way. The homogenates were strained and put it in the 15 mL of sterile propylene tubes, and centrifuged at 2500 rpm for 5 minutes at 4°C. Finally, the pellet resuspended 1 mL of sterile PBS.

Flowcytometry Analysis

To analyze the mixture of *M. oleifera* and Albumin extract against pro-inflammatory cytokines, the isolated cells were taken for 200 µL and placed on the sterile microtubes and centrifugated at 2500 rpm for 5 minutes at 4°C. Subsequently, the supernatant was separated and added with 40 µL of antibody staining and incubated for 15-20 minutes in the icebox with the dark condition. The cells then were added with 300 µL of PBS sterile and placed into flow cytometry for analysis.

Data Analyis

The data were analyzed using one-way ANOVA with significance level $p < 0.05$ using SPSS to determine the relationship between variables, and then continued with a Tukey test to find the most involving variable.

RESULTS AND DISCUSSION

Moringa oleifera and Albumin Decreased the Relative Number of T Cells

The administration of *M. oleifera* and Albumin against DM on the Balb/c mice showed no significant result. The relative number of CD4+ CD8+ increased compared to DM positive control. This research tested three groups of different doses of *M. oleifera* and VipAlbumin® and compared them to negative control and DM

positive control. We found that Dose 1 contains 100 mg.kg^{-1} body weight *M. oleifera* + 416.25 mg.kg^{-1} bodyweight VipAlbumin® gave a result ($p<0.05$), but it is not significant compared to DM positive control (Fig. 1).

The number of CD4+CD8+ cells on the positive control number showed an increasing number ($p<0.05$) compared to healthy mice from 1.84% to 2.52%. Relatively, which given *M. oleifera* and VipAlbumin® orally showed no significant decrease. Dose 1 became 1.43%, Dose 2 became 1.87%, and Dose 3 became 1.56% (Fig. 1). Based on the result shown by flowcytometry analysis, the conclusion is, *M. oleifera* and VipAlbumin® did not give a specific result to reduce the relative number of CD4+ CD8+.

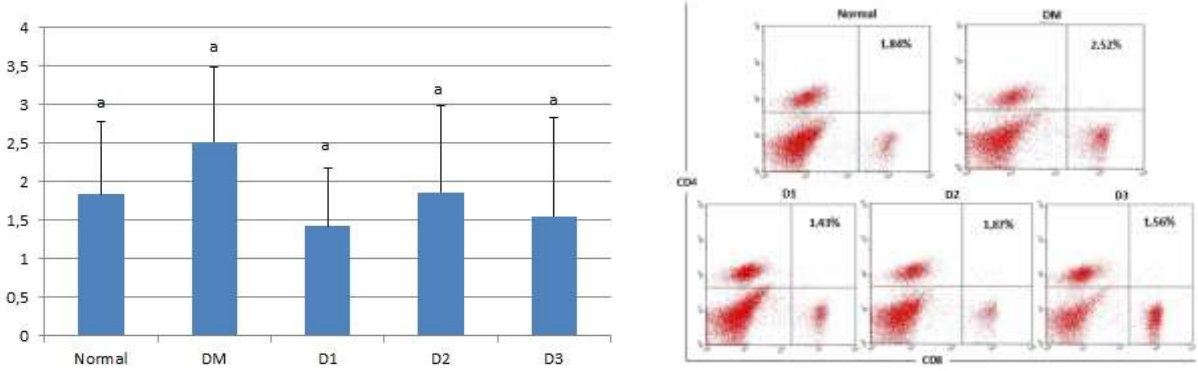

Figure 1. The Administration of *Moringa oleifera* and Albumin increased a relatively number but not significant of T cells. **Normal** : a healthy group, **DM** is positive control induced with 145 mg.kg^{-1} body weight STZ, **D1** : dose one with STZ injection and 100 mg.kg^{-1} *Moringa oleifera* + 416.25 mg.kg^{-1} VipAlbumin®, **D2** : dose 2 with STZ injection and 150 mg.kg^{-1} *Moringa oleifera* + 208.15 mg/kg VipAlbumin®, **D3** : dose 3 with STZ injection and 50 mg.kg^{-1} *Moringa oleifera* + 624.375 mg.kg^{-1} VipAlbumin®.

Moringa oleifera and Albumin Suppressed the Pro-Inflammatory Cytokines

As a pro-inflammatory role, TNF-α is a contributor to β-cell destruction. The result showed a decrease in inflammation of TNF-α produced by CD4 in healthy mice was 3,76%. After being injected by STZ, the number of inflammation of TNF-α produced by CD4 increased became 4.21% but not significant ($p > 0.05$) (Fig. 2b).

The administration of *Moringa oleifera* and VipAlbumin® in D1, D2, and D3 groups decreased the inflammation number significantly ($p < 0.05$). The result showed that the inflammation number of TNF-α produced by CD4 T cells for Dose 1 became 1.95%, Dose 2 became 3.47%, and Dose 3 became 0.96% compared to DM positive, which 4.21%.

IFN-γ produced by CD4 T cells seems important in susceptibility and progression of DM. This study result showed a decrease in the inflamation number of IFN-γ in control positive DM compared to healthy mice (normal). The inflammation number of healthy mice was 2.43% became 2.05% but not significant ($p>0.05$).

The inflammation number of changed following the administration of *M. oleifera* and VipAlbumin® showed both a decrease and an increase. A significant decrease of inflammation number of IFN-γ produced by CD4 T cells was observed in D1 ($p<0.05$) compared to DM positive control was 1.39%. While in D2, it has an increased inflammation number but not significant ($p>0.05$) DM positive control was 2.3%. For D3, it has a decrease inflammation number significantly ($p<0.05$) compared to DM positive control was 1.56%.

Moringa oleifera and Albumin Decreased the Relative Number of T Cells

Flowcytometry analysis results for B220 cells showed both a significant increase and a decrease (Fig. 3). The result found that the relative number of B220 cells of the positive control DM was 8.6% compared to healthy mice 12.82%. The relative number of B220 cells showed significant ($p<0.05$) difference on both decrease and increase result after given *M. oleifera* and VipAlbumin® orally. A significant increase observed on D1 ($p>0.05$) compared to DM positive control was 10.03%. While on D2, a significant increase observed and compared to DM positive control, was 23.67%. On D3, a different result showed. A significant decrease observed on D3 ($p<0.05$) compared to DM positive control was 4.2%.

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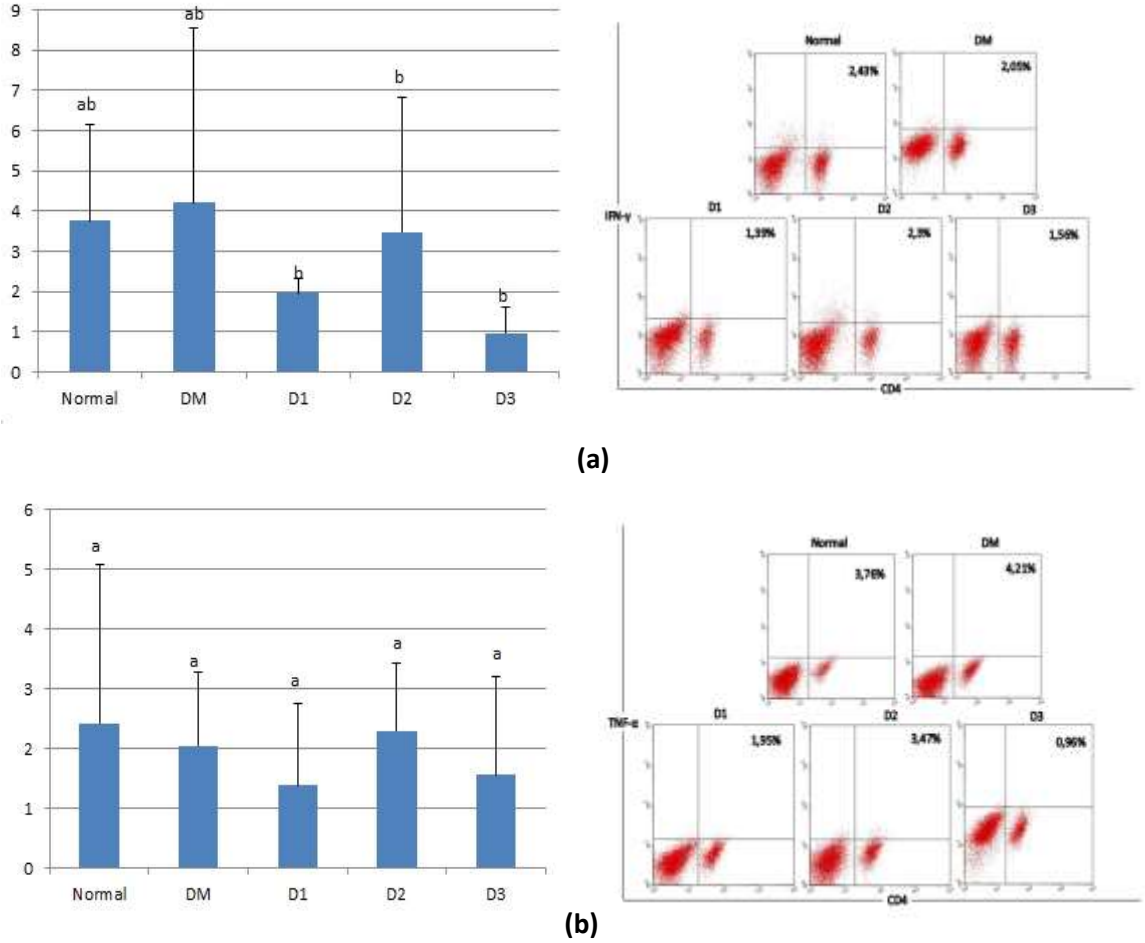

Figure 2. (a) Production profile of TNF-α Cytokines by CD4 cells with T cell CD4 TNF-α total percentage. (b) Production profile of IFN-γ Cytokines by CD4 cells with CD4 IFN-γ total percentage. (**Normal** : a healthy group, **DM** is positive control induced with 145 mg.kg^{-1} body weight STZ, **D1** : dose one with STZ injection and 100 mg.kg^{-1} *Moringa oleifera* + 416.25 mg.kg^{-1} VipAlbumin®, **D2** : dose 2 with STZ injection and 150 mg.kg^{-1} *Moringa oleifera* + 208.15 mg/kg VipAlbumin®, **D3** : dose 3 with STZ injection and 50 mg.kg^{-1} *Moringa oleifera* + 624.375 mg.kg^{-1} VipAlbumin®)

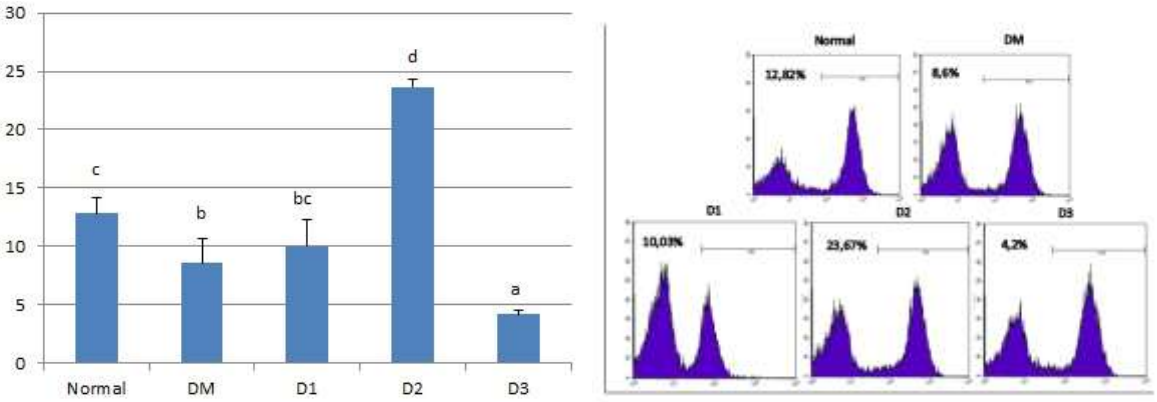

Figure 3. Result graphic calculation for B220. (**Normal** : a healthy group, **DM** is positive control induced with 145 mg.kg^{-1} body weight STZ, **D1** : dose one with STZ injection and 100 mg.kg^{-1} *Moringa oleifera* + 416.25 mg.kg^{-1} VipAlbumin®, **D2** : dose 2 with STZ injection and 150 mg.kg^{-1} *Moringa oleifera* + 208.15 mg/kg VipAlbumin®, **D3** : dose 3 with STZ injection and 50 mg.kg^{-1} *Moringa oleifera* + 624.375 mg.kg^{-1} VipAlbumin®)

Discussion

This study used *M. oleifera* and Albumin as anti-diabetics to reduce inflammation and blood glucose levels on a diabetic Balb/c mice model. *Moringa oleifera* contains many important biological substances such as flavonoid pigments like kaempferol, rhamnetin, isoquercitrin, and kaempferitrin.

Several phytochemicals become a particular interest because of their medicinal function [13]. Phytochemical investigations of *M. oleifera* have revealed the presence of 4-(4'-o-acetyl-α-L-rhamnopyranosyloxy) benzyl isothiocyanate, niazimicin, pterygospermin, benzyl isothio-cyanate, and 4-(α-L-rhamnopyranosyloxy) benzyl glucosinolates [14].

Furthermore, the previous study also reported that flavonoids act as insulin mimetic or insulin secretagogues by influencing the pleiotropic [14]. They also proposed that Albumin is necessary for the proper distribution of body fluids between intravascular compartments and body tissues and functions as a plasma carrier by binding several hydrophobic hormones. Albumin can maintain the blood from leaking out from blood capillaries, after being treated by STZ.

Lymphocyte cells, such as CD4+CD8+, has been known to play an essential role in obesity and obesity-induced insulin resistance [3]. Furthermore, this research explained that $CD4^+$ effector T cells could be further divided into pro-inflammatory Th17, Th1, Th2, and regulatory T cells ($CD4^+CD25^+Foxp3^+$). This division is closely related to its functionality and the production of cytokine. Th1 cells could produce interleukin-2 (IL-2) and tumor necrosis factor-beta (TNF-β), interferon-gamma (IFN-γ), triggering phagocyte-dependent inflammation and cell-mediated immunity. CD4 T cells produce TNF-α molecule as potent inflammatory mediator. TNF-α also produced by monocytes, macrophages, $CD8^+$ T cells, B cells, endothelial cells, NK cells, and lymphokine-activated killer (LAK) cells [15].

Interferon-γ produced by lymphocytes as a result of the activation by specific antigens or mitogens. INF-γ is a potent activator of macrophages, and thus, it has important immunoregulatory functions [2].

The result of this study showed that the relative number of T cells decreased significantly ($p<0.05$) after the administration of *M. oleifera* and VipAlbumin® compared to DM positive control from 2.52% to 1.43% for Dose 1. The administration of *M. oleifera* and VipAlbumin® also decreased pro-inflammatory cytokines significantly ($p<0.05$). This study showed that TNF-α produced by CD4 T cells decreased compared to DM positive control in Dose 3, from 4.22% to 0.96%.

Meanwhile, IFN-γ produced by CD4 T cells gave decrease results compared to DM positive control in Dose 1, which contains 100 $mg.kg^{-1}$ body weight *Moringa oleifera* + 416.25 $mg.kg^{-1}$ body weight Albumin, from 2.05% to 1.39%. On the other hand, the IL-6 which is produced by isolated mouse islets after being exposed to interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) gave a significant result after the administration of *M. oleifera* and Albumin, on Dose 3 from 8,6% to 4,2%.

CONCLUSION

In this research, *Moringa oleifera,* combined with Albumin, gave a result that could not be used as an anti-inflammatory. This result happened because the dose used here was low. *Moringa oleifera* and VipAlbumin® can be used to cure the inflammation in DM, but with another dose, which is not part of this research. The activity of *M. oleifera* and VipAlbumin® with the right dose was able to decrease the relative number of a proinflammatory cytokine such as TNF-α, IFN-γ, produced by T lymphocytes or macrophages.

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Phytochemical Screening by FTIR Spectroscopic Analysis and Antibacterial Activity of *Sesbania grandiflora* Fraction Against *Edwardsiella tarda*

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Abstract

Sesbania grandiflora is a medicinal plant that has been used by many Indonesian people. They have the ability as antibacterial because they contain bioactive compounds that inhibit bacterial growth. *Sesbania grandiflora* also contains other active compounds such as tannins, saponins, steroids, flavonoids, and terpenoids. This study aims to determine the antibacterial activity of extracts, n-hexane fraction, ethyl acetate fraction and water fraction of *Sesbania grandiflora* against *Edwardsiella tarda* using the well diffusion method in different concentration (75 ppm, 150 ppm dan 225 ppm), and knowing the active compounds of FTIR absorption bands. MIC (*minimum inhibitory concentration*) value of extract, n-hexane, ethyl acetate, and water fractions respectively was 0.51 %, 1.11 %, 0.97 %, dan 0.79 %. inhibitory zone diameter of the n-hexane fraction is most active against *Edwardsiella tarda* bacteria. The results of FTIR identification showed that n-hexane fraction contained flavonoids and terpenoids, as seen from absorption bands that function as antibacterial compounds.

Keywords: antibacterial, *Edwardsiella tarda,* FTIR, Phytochemical *Sesbania grandiflora.*

INTRODUCTION

Increasing aquaculture production programs carried out during the period 2010-2014 has shown tangible results with an average increase of 23.8% per year. In 2010 aquaculture production was 6.27 million tons. Whereas in 2014, it rose to reach 14.52 million tons. The value of temporary production in 2014 was Rp. 109.78 trillion [1,2]. However, aquaculture development still faced with several global challenges and problems. One of the problems in aquaculture development is the development of fish and environmental health systems, where the disease is the main obstacle [3].

The fish disease is anything that can cause interference with fish, both directly and indirectly. Many factors determine which fish become sick. The main factor is the host, pathogens (microbes, parasites), and the environment that involves physical, chemical, or behaviors such as stress [4]. Where in aquaculture is inseparable from bacterial pathogenic infections that cause the death of fish at this time. One of the infectious diseases was caused by the bacterium *Edwardsiella tarda*, which causes Edwardsiellosis or Emphisemathous Putreactive Disease of Catfish (EPDC). *Edwardsiella tarda* is a gram-negative bacteria belonging to the cocci-shaped *Edwardsiella* genus with a size (0.3-1.2) x (1.0-0.3) µm, *E. tarda* is an *Edwardsiella* causative agent [5], which can cause death in fish, both freshwater and seawater habitats. These bacteria can attack fish when fish experience stress, which is caused by high stocking density, poor water quality conditions, and high organic matter content [6]. Thus, *E. tarda* can cause a decrease in the productivity of fish farmers, which can also cause harm to farmers. Edwardsiellosis or Emphisemathous Putreactive Disease of Catfish (EPDC) caused by *E. tarda* is a disease that has been known as a major disease in catfish cultivation in America. *E. tarda* infections in various types of fish that live in free waters have been reported, such as channel catfish, eels, mullet, Chinook salmon, flounder, carp, tilapia, and striped bass [7].

Cultivators generally prevent fish diseases by giving various antibiotics, such as ampicillin, tetracycline, and disinfectants. The continuous use of antibiotics if the application is not right can cause pathogenic bacteria to become resistant. There is a buildup of drug residues in the body of the fish and the aquatic environment, which in turn can endanger the consumers of aquaculture products [7]. This study aimed to determine the antibacterial activity of extracts, n-hexane fraction, ethyl acetate fraction, and water fraction of *Sesbania grandiflora* against *Edwardsiella tarda*, and

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knowing the active compounds of FTIR absorption bands.

MATERIAL AND METHOD

Leaves Preparation

The leaves of *Sesbania grandiflora* obtained from the Sumba area, East Nusa Tenggara. The leaves of *S. grandiflora* used are old leaves, washed using heated water, drained, and air-dried. The dried leaves are then blended and sifted using a sieve to obtain the desired turi leaf powder.

Extraction

Extraction process using the method of maceration with Ethanol solvent (3:1 w/v). This process carried out for 48 hours. The longer the extraction time, the more extracted extract obtained.

Fractionation

Ethanol extract obtained from maceration in the form of a paste, dissolved in 75 mL of distilled water, then separated in a separating funnel with n-hexane 75 ml added partitioned three times, then separated into n-hexane fraction. The residue from n-hexane fractionation separated from the separated species by adding ethyl acetate solvents and partitioned as much as three times. Fractionation results concentrated using a rotary evaporator, so that the n-hexane fraction, ethyl acetate fraction, and water fraction obtained. The extract used to inhibit or kill the growth of *E. tarda* bacteria in several concentrations, namely 75 ppm, 150 ppm, and 225 ppm.

Antibacterial activity test

Edwardsiella tarda bacterial suspension originates from the Central Java Jepara Quarantine Hall. Determination of the antibacterial activity of *E. tarda* was conducted by using the well method (by means of the media using a well). Then each hole filled with extracts and a fraction of 25 microlites with a dose concentration of 75 ppm, 150 ppm, and 225 ppm, which diluted in 10% Dimethyl sulfoxide (DMSO).

Determination of MIC (*Minimum Inhibitory Concentration*) and MBC (*Minimum Bactericidal Concentration*) used published methods by Mohanty and Sahoo [8] by using data from testing antibacterial activity at several concentrations of *S. grandiflora*. Determination of the MIC value is done through Ln Mo (Ln concentration of *S. grandiflora* leaves) on the X-axis against the square of the inhibiting Zone (Z^2) on the Y-axis. The intersection between the equations obtained from linear regression Y = a + bX with the X-axis is the Mt value of *S. grandiflora* leaf fraction concentrates at Y = 0). The Mt value is the MBC value, while the MIC value is 0.25 X Mt.

RESULT AND DISCUSSION

Antibacterial activity

Testing the antibacterial activity of extracts, n-hexane fraction, ethyl acetate, and water at several concentrations aims to know the inhibitory concentration of the inhibitory power and the potential of *S. grandiflora* leaves as a natural antibacterial source. The results of testing the antibacterial activity showed that the extract, n-hexane fraction, ethyl acetate, and water had antibacterial activity against *E. tarda* bacteria indicated by the presence or absence of inhibitory zones. The results of the antibacterial activity of extracts and fractions of *Turi* leaves (*S. grandiflora*) against *E. tarda* bacteria (Fig. 1)

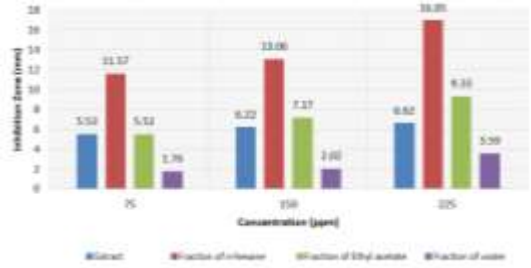

Figure 1. Antimicrobial Activity of Turi Leaves *(S. grandiflora)*

The results showed that the concentration of n-hexane fraction was better in inhibiting the growth of *E. tarda* compared to ethanol extract, ethyl acetate fraction, and water fraction, with a large inhibition zone of 16.95 mm at a concentration of 225 ppm. The inhibition zone was not much different from the following two concentrations of 13.56 mm and 11.57 mm. It is probably due to the content of the active compound of *Turi* leaves, which inhibits bacterial growth and can even kill bacterial cells and the n-hexane fraction containing the compounds contained therein may be an alkaloid, which has antibacterial activity [9].

The well method test showed that the largest inhibition zone was the n-hexane fraction, which could produce a 14.32 mm inhibition zone of *E. tarda* with a concentration of 125 ppm, where higher concentration showed more significant inhibition zone (Fig. 1). The ability of *Turi* leaves to inhibit the growth of bacterial species has been reported. *Turi* leaves can inhibit the growth

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of *Escherichia coli*, *Bacillus susbtilus*, *Staphylococcus aureus* [10].

The antibacterial inhibition of *Turi* leaves is caused by antibacterial compounds [10]. *Turi* leaves are plants that have been used as medicine because they contain active ingredients such as flavonoids, alkaloids, saponins, tannins, steroids, and terpenoids [11].

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC and MBC are the minimum concentrations to kill the growth of microorganisms. The results of the concentration of the minimum inhibitor extract, the n-hexane, ethyl acetate, and water fraction against the growth of *E. tarda* bacteria showed in Table 1.

Table 1. Growth of *E. tarda* bacteria during the research

Fraction	MIC (%)	MBC (%)
Extract	0.51	2.02
n-hexane fraction	1.11	4.43
Etil acetate fraction	0.97	3.87
Water Fraction	0.79	3.16

Based on the results of the wells dilution test, the MIC value of the n-hexane fraction is 1.11%. It is indicates that the n-hexane fraction can inhibit the growth of E.tarda. While the MIC value of the extract, ethyl acetate, and water fractions can inhibit *E. tarda* growth, but the inhibitory zone value is small. The MBC value also showed the highest value was the n-hexane fraction and followed by the ethyl acetate, water, and extract fractions for 4.43%, 3.87%, 3.16%, and 2.02%, respectively. It shows that the content of *S. grandiflora* leaves, which is purified by the active compounds contained, can inhibit and kill *E. tarda* growth. It was explained that the compounds in non-polar and semi-polar fractions could attract polar compounds, such as flavonoids, alkaloids, and terpenoids, that can reduce bacterial growth [12].

FTIR Analysis

FTIR spectroscopy is a tool used to identify compounds, precisely organic compounds, both qualitatively and quantitatively. The analysis carried out by looking at the shape of the spectrum by looking at specific peaks that show the functional groups possessed of the compound [13]. The results of FT-n-hexane fraction analysis can be seen in Figure 2.

The results of the FTIR spectra of *Sesbania grandiflora* n-hexane fraction (Table 2) showed a range of O-H form intermolecular hydrogen bonds with small intentions at wave number 3504.104 cm^{-1} which assumed to be a phenol compound. Uptake that appeared on 2940 - 2850 with wavenumber 2924.387 cm^{-1} is absorption from CH aliphatic. Absorption at 1450,696 cm^{-1} is a C = C aromatic double bond belong to the flavonoid compound. Absorption band in absorption area 1490 - 1400 is a functional group OH RCO2H, which is an alcohol group. So, it assumed that in the n-hexane fraction, it contains terpenoids. Flavonoid compounds were shown in functional groups, namely aliphatic C-H groups and C = C aromatic double bonds [14]. Terpenoid compounds are compounds that have alcohol functional groups, carboxylic acids [15].

The results of the antibacterial activity test showed n-hexane fraction as the most active fraction, which had the largest inhibition zone, compared to the extract, ethyl acetate, and water fraction. Terpenoid compounds, flavonoids are organic compounds that very beneficial for health, namely as an anti-inflammatory, antibacterial, antioxidant, and even as an ingredient to increase endurance. Flavonoid terpenes found in herbal plants can damage bacterial membranes by destroying the outer membrane of Gram-negative bacteria [16-18].

Figure 2. Analysis of n-hexane fraction *Sesbania grandiflora*

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Table 2. Isolate and Cluster absorption band numbers in the n-hexane fraction

Absorption and Compound Ribbon Numbers	Absorption Ribbon Numbers (cm^{-1}) [3,19]	Chemical Structure	Chemical Name	Source
3550 - 3230	3504.104	OH		[20]
2940 - 2850	2924.387	C-H aliphatic	Alkane	
1650 - 1460	1450.696	C=C aromatic		
1490 - 1400	1383.101	O-H RCO_2H	Alcohol (H-bounded) carboxylic acid	

CONCLUSIONS

The results showed that *S. grandiflora* extract activity of n-hexane fraction, ethyl acetate fraction, and water fraction using wells diffusion method was 75 ppm, 150 ppm, and 225 ppm, as well as knowing the active compounds of FTIR absorption bands. MIC (Minimum inhibitory concentration) extract, n-hexane, ethyl acetate, and water fractions respectively 0.51%, 1.11%, 0.97%, and 0.79%. Inhibition zone diameter of n-hexane fraction is the most active against *E. tarda* bacteria. The identification of FTIR showed that the n-hexane fraction contained flavonoids and terpenoids, as seen from the absorption bands formed.

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The Potential Use of Microorganisms as Degradation Agent on Naphthalene and Phenanthrene

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Abstract

This study aims to find the most appropriate indigenous bacteria as Naphthalene and Phenanthrene degrading agents carried out in the growth response test. This research is very important to find the best bacterial agent, which in the future can reduce the impacts of oil pollution, as well as from the presence of oil sludge Bacterial cultures added in the media as much as 5% (v/v), which had previously been measured OD660nm = 0.5. The growth response test was carried out by growing 20 mL of bacteria in AMS media which was added with PAH substrate, naphthalene or Phenanthrene in various concentrations: 200 ppm, 500 ppm, 800 ppm, and 1000 ppm. In the naphthalene substrate with 24-hour incubation, the best treatment was in isolates A with a concentration of 200 ppm. In the phenanthrene substrate 24 hours incubation, the best treatment was in isolate A at 200 ppm, and at 48 hours incubation. Bacterial isolates F in 24-hour incubation increased growth in all treatments and controls. The bacterial E isolate on the phenanthrene substrate at 24-hour incubation has increased growth, which occurs until the incubation time is 48 hours, both in the control and phenanthrene substrate. E Isolate is tolerant of increasing substrate concentrations up to 1000 ppm, both naphthalene and phenanthrene and has the best growth ability. It can be concluded that isolate E has the best growth ability and is tolerant of increasing substrate concentrations up to 1000 ppm. E isolates were designated as the best isolates of naphthalene and phenanthrene degradation.

Keywords: Dumai, Growth Response, Naphthalene, Phenanthrene

INTRODUCTION

Pollution is one of the important problems both pollution of organic matter and inorganic materials. Pollution of organic matter, which gets the most attention, is hydrocarbon pollution because of its abundance. Oil sludge is a precipitate formed at the bottom of the storage tank due to contact between oil, air, and water. Increasing the accumulation of oil sludge can cause a reduction in oil storage capacity and accelerate the occurrence of the rusting process [1,2]. In addition, there are aliphatic, aromatic, and polyaromatic hydrocarbons (PAHs) in oil sludge. PAH content in oil sludge reached 13.24% [3]. These compounds are one of the concerned pollutant sources because they are carcinogenic, mutagenic, and immuno-toxic. The Environmental Protection Agency in the United States categorizes 16 types of PAHs as the main environmental pollutants [4,5,6]. Types of PAHs that have two or three rings (naphthalene and phenanthrene) are compounds that easily degraded compared to other types of PAH [7].

As one of the largest petroleum refining locations in Indonesia, Dumai City, Riau Province, is inseparable from the impacts of pollution and the presence of oil sludge and harmful compounds inside. This study aims to find the most appropriate indigenous bacteria as Naphthalene and Phenanthrene degrading agents carried out in the growth response test. This research is very important to find the best bacterial agent, which in the future can reduce the effects of oil pollution as well as from the presence of oil sludge.

MATERIAL AND METHOD

Research was carried out in Laboratorium Terpadu, Biology department, Faculty of Science and Technology, Airlangga University in November 2018-July 2019. The bacteria used in this study were seven isolates of pure indigenous bacteria from Dumai oil sludge, which obtained from previous research and coded A (4), A (4) 2, Ao (5) 2, A (5) 3, A (5) 5, A (6) 1, A (6) 2, which are then sequentially called isolates A, B, C, D, E, F, G. The media used in this study are Nutrient Broth, Nutrient Agar, and Synthetic Mineral Water (AMS), which was a modified composition [8].

Bacterial cultures added in the media as much as 5% (v/v), which previously had been measured OD660nm=0.5. The culture incubated for three days (72 hours) with a shaker speed of 120 rpm at room temperature. The data obtained in the form of bacterial growth response through measurement of OD every 24 hours using a spectrophotometer and TPC at 72 hours. OD measurement intended to determine the growth

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of total bacterial biomass as indicated by increasing substrate turbidity. The TPC method carried out to confirm and count the number of living bacterial colonies.

The growth response test carried out by growing 20 mL of bacteria in AMS media, which added with PAH substrate, naphthalene, or phenanthrene, with various concentrations of 200 ppm, 500 ppm, 800 ppm, and 1,000 ppm. Bacterial cultures added in the media as much as 5% (v/v), which previously had been measured OD660nm=0.5. Furthermore, the culture incubated for 72 hours with a shaker speed of 120 rpm at room temperature. The data obtained were in the form of bacterial growth response through measurement of OD every 24 hours using a spectrophotometer and carried out TPC at 72 hours using Nutrient Agar media. We also conducted a one-way ANOVA and Duncan statistics test to find out the difference between the treatment of the substrate treatment concentration and the incubation time.

RESULT AND DISCUSSION

We use bacterial isolates A, E, and F based on previous research. The bacterial growth response test carried out on naphthalene and phenanthrene substrates in different cultures with different concentrations: 200, 500, 800, 1000 ppm [9]. There is a growth response of bacteria A, E, and F on naphthalene substrates with different concentrations observed for 72 hours (Fig. 1), and the results on phenanthrene substrates (Fig. 2).

Bacteria Growth on Naphthalene Substrates

Bacteria grown on naphthalene substrates show a varied growth response (Fig. 1). The bacterial isolate A at the 24-hour incubation period shows the log (exponential) phase. It proved by the value of OD, which becomes very high at 24 hours incubation time. At 48 hours incubation, bacterial A OD decreased in control, 800 ppm and 1,000 ppm naphthalene concentration treatment. It continued to decrease until 72 hours incubation, while 200 ppm and 500 ppm concentration treatment increased up to 72 hours, and continued to increase. It happens because, at 800 ppm and 1,000 ppm naphthalene concentration, it becomes an inhibitor concentration that can inhibit cell growth because it is toxic [10].

In the 24 hours incubation of bacterial isolates, there was an increase in growth, both in the control and treatment of concentration. This increase in growth occurred until 48 hours incubation for the treatment of concentrations of 200 ppm to 1,000 ppm, while in the control of bacterial growth began to decline. In the control, the OD value dropped because there was no carbon source for bacterial growth. The same thing also happened at 72 hours incubation, where growth continued to increase in the concentration treatment, while in the control there was a continuous decline.

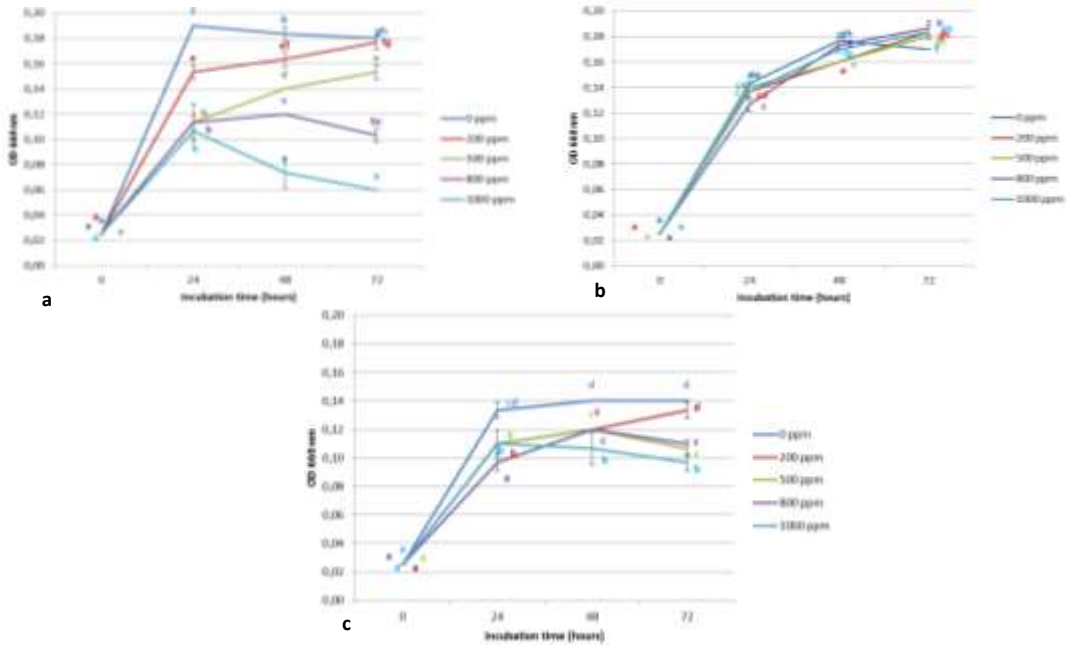

Figure 1. Response to Growth of Bacterial Isolates on the Naphtalene substrate with different concentrations
Description: a) Isolates A, b) Isolates E, and c) Isolates F

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Bacterial isolates F in 24-hour incubation increased growth in all treatments and controls. During the 48-hour incubation period, there was a varied growth: in the treatment concentration of 200 ppm and control, there was an increase in growth, whereas in naphthalene 500 ppm, 800 ppm and 1,000 ppm there was a decrease in OD values up to 72 hours incubation. At the treatment of 200 ppm at the incubation time of 72 hours, bacterial growth continues to increase, while in the control, growth has entered the stationary phase.

Bacterial Growth on Phenanthrene Substrates

The bacterial growth response on phenanthrene substrates with different concentrations showed in Figure 2. The bacterial isolate A at 24 incubation time reached the log phase. At 48 hours incubation time in phenanthrene 200 ppm and 500 ppm, there was still an increase in growth, whereas in concentrations of 800 ppm and 1,000 ppm there was a decrease, and in the control entering the stationary phase.

This happened until 72 hours incubation. At phenanthrene concentrations of 800 ppm and 1,000 ppm, recorded a decrease in growth. It happened because, at this level, it reached an inhibitor concentration that inhibits bacterial growth. While a decrease in control has occurred because the media has no carbon source for bacterial growth.

The classic problem with microbiology is that bacteria exhibit two types of growth when they are in a culture of mixing two carbon sources. In the case of co-utilization, there is a model that can predict each carbon source in the supply of amino acids [11].

The bacterial E isolate on the phenanthrene substrate at 24-hour incubation has increased growth. It occurred until the incubation time of 48 hours, both in the control and phenanthrene substrate. At 72 hours of incubation, in the treatment of variations in concentration, bacterial growth still increased while in the control decreased. It happened because, in the control, there is no carbon source that can be used by bacteria for growth whereas in the treatment of phenanthrene concentration variations there are still carbon sources to grow [12].

There was a growth of bacterial isolates F at 24 hours incubation time in various treatments and controls. At 48 hours incubation, the addition of phenanthrene substrate with a concentration of 200 ppm increased while the addition of 500 ppm, 800 ppm, 1,000 ppm, and control decreased. At 72 hours of incubation, all treatments experienced a decrease in growth, both in the control and various concentration of the phenanthrene substrate. It happened because the age of the bacteria reaches the Lag phase. This phase shows that bacteria cannot duplicate or modify their physiological performance to cope with their environmental influences [13].

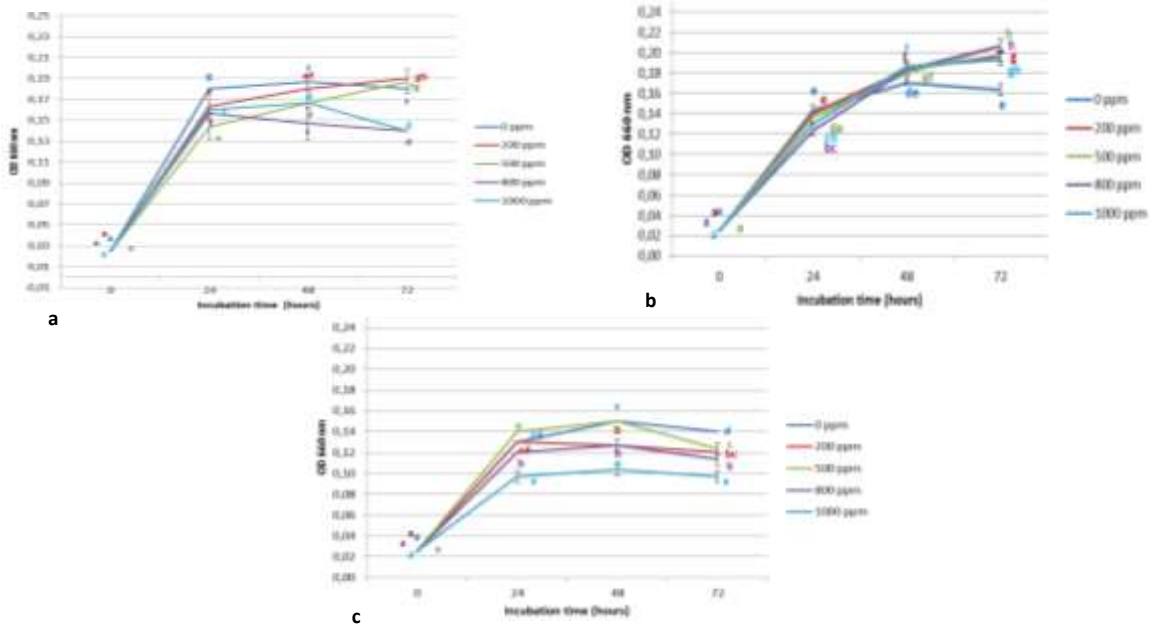

Figure 2. Response to Growth of Bacterial Isolates on the Phenanthrene Substrate with Different Concentrations
Description: a) Isolates A, b) Isolates E, c) Isolates F

OD and TPC values

To strengthen OD measurement data, we measured TPC in log CFU.mL^{-1} at 72 hours incubation time, then compared OD values and TPC values at 72 hours incubation time presented in Figure 3. Based on the one-way ANOVA statistical test followed by the Duncan test, it produces distinguishing notations between the treatment of substrate concentration and incubation time (Fig. 1 and Fig. 2).

In the naphthalene substrate with 24-hour incubation, the best treatment was in isolates A with a concentration of 200 ppm (there were significant differences). However, at 48 and 72 hours incubation, the best treatment was E isolates at a concentration of 800 ppm. In the phenanthrene substrate 24 hours incubation, the best treatment was in isolates A at 200 ppm, and at 48 hours incubation, isolates E at 1000 ppm was the best treatment, but not significantly different from treatment E at 800 ppm. At the incubation time of 72 hours, the treatment of isolates E at 800 ppm was the best, but not significantly different from E treatment at 500 ppm.

Based on its growth response, bacterial isolates E tend to be resistant to variations in substrate concentration, both naphthalene, and phenanthrene. The physical-chemical properties are one of the determinants of the ease of accessibility of hydrocarbons by microbes, such as type, form, solubility, and toxicity [9,14].

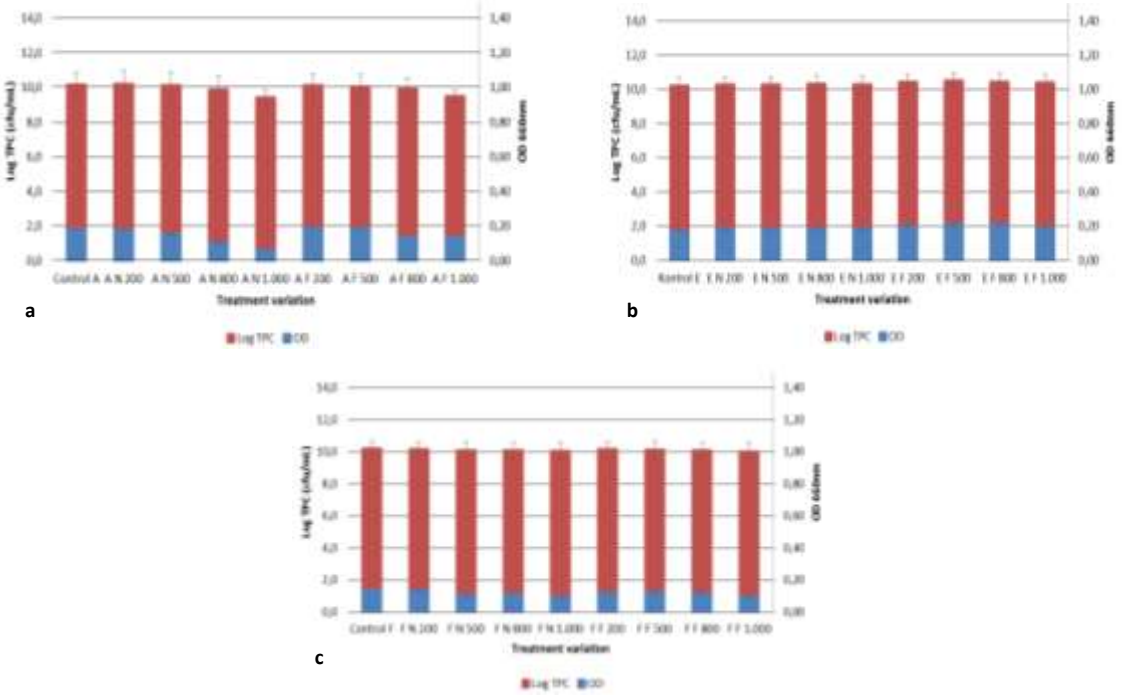

Figure 3. Histogram Comparison of OD and TPC values for N Naphthalene and Phenanthrene at 72 hours incubation period
Description: a) Isolates A, b) Isolates E, c) Isolates F

CONCLUSION

From the results of the growth response test, it concluded that isolate E has the best growth ability and is tolerant of increasing substrate concentrations up to 1000 ppm. Therefore, E isolates designated as the best isolates for naphthalene and phenanthrene degradation.

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Analysis Activity of *Elephantopus scaber* Leaves Extract Against Quantitative Changes of Lymphocytes Cells in BALB/c Mice After Induction of DMBA and Estrogen

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Abstract

The carcinogenic compounds such as DMBA known for having the ability to promote the DNA damage that affects the growth regulator genes. Therefore, uncontrolled cell growth will occur and change the immune system. *Elephantopus scaber* extract is considered as an alternative way of handling breast cancer since it capable to affect the work of the immune system. Hence, this study aims to determine the effect of *E. scaber* extract on quantitative changes of $CD4^+$, $CD8^+$ T cells and proinflammatory cytokines $CD4^+IFN\text{-}\gamma^+$ and $CD4^+TNF\text{-}\alpha^+$ after induction of DMBA and estrogen. In vivo experiments were carried out by using 5-6 weeks old of female mice BALB/c strain. The extract of *E. scaber* leaf powder was obtained from Material Medica Batu, Malang. The experimental animals were divided into five groups such as negative control group (K-), positive control group (K+), mice treated with *E. scaber* extract for a week (group 1), mice treated with *E. scaber* extract for 2 weeks (group 2), and mice treated with *E. scaber* extract for 3 weeks (group 3). Induction of DMBA and estrogen was conducted by injection of DMBA (7.12 dimetylbenz (α) anthracene) 0.56 $mg.kgBW^{-1}$ and estradiol hormone 0.0504 $mg.kgBW^{-1}$. Mice were treated with *E. scaber* extract with a dose of 50 $mg.kgBW^{-1}$. Lymphocytes were isolated from spleen and observed the $CD4^+$, $CD8^+$ T cells and proinflammatory cytokines $CD4^+IFN\text{-}\gamma^+$ and $CD4^+TNF\text{-}\alpha^+$ T cells through flow cytometry analysis. One-way ANOVA ($p < 0.05$) and SPSS were used to analyze this data. The results showed that the induction of DMBA and estradiol hormone affected mice fur loss. It also decreased the immune system by lowering the number of CD4 and CD8 T cells. However, *E. scaber* extracts increased the number of pro-inflammatory cytokines TNF-α. Furthermore, the DMBA and estradiol induction also promoted the number of $CD4^+IFN\text{-}\gamma^+$ in the first week, but decreased the number of $CD4^+IFN\text{-}\gamma^+$ at weeks 2 and 3, then increase the number of $CD4^+IFN\text{-}\gamma^+$. So, the treatment of *E. scaber* extracts demonstrated the ability to restore the homeostasis of the immune system in the first and second weeks.

Keywords: $CD4^+$, $CD8^+$, DMBA, estrogen, *E.scaber,* $IFN\text{-}\gamma^+$, $TNF\text{-}\alpha^+$

INTRODUCTION

DMBA compounds (7.12 dimetylbenz (α) anthracene) are carcinogenic substances that have a higher and more stable potential for creating cancer models in experimental animals. DMBA compounds induced in the body will be metabolized in the liver by cytochrome P450 CYP1A1 or CYP1B1 and mEH (microsomal epoxides hydrolase) in peripheral tissues forming covalent bonds to DMBA 3,4 diol-1,2 epoxide (DMBA-DE) to DNA adduct form [1].

Among various kind of cancer, breast cancer is being the most common cancer suffered by women [1]. According to the data, breast cancer is the second most common disease after lung cancer in Indonesia [2].

The presence of cancer will cause a change in the immune system. Commonly, cancer cells cannot be recognized by the immune system, because of cytokine production that prevents the immune response such as the TGF-β. The increased level of TGF-β cytokines will inhibit the work of CD4 and CD8 T cells [3]. Those cytokines will be needed for the development of CD8 + cytotoxic cells to become effector cells [4].

Immune cells that reside around the cancer cells have been known to kill cancer cells around them. Some lymphocyte subpopulations, both T helper lymphocytes and cytotoxic T play a role in eliminating tumor antigens. Cells that contain tumor antigens will express their antigens together with class I MHC molecules that form a complex through TCR (T cell receptor) from cytotoxic T cells [4].

One of the alternative treatments is the utilization of herbal medicines. Cancer prevention through medicinal plants is based on the chemopreventive compounds that can inhibit the carcinogen process so that it can prevent the cancer growth [5]. Flavonoids and isoflavonoids had widely known to inhibit the process of carcinogenesis [6].

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Elephantopus scaber or *Tapak Liman* is one of herbal that has the potential ability to be used as herbal medicine. *Tapak Liman* plant containing several compounds such as flavonoids, alkaloids, tannins, phenols, steroids, terpenoids, saponins, isodeoxyelephantopin, and also deoxyelephantopin. Deoxyelephantopin and isodeoxyelephantopin have been reported to have the ability to induce apoptosis and inhibit the activation of Nf-kB in cancer cells [7,8].

Elephantopus scaber extract has a function as an anti-inflammatory, analgesic, antioxidant, and antimicrobials. Flavonoids can prevent tumorigenesis through several mechanisms, namely cytochrome P450 isoenzyme activity, induction of glutathione S-transferase enzyme (GST), and oxidative inhibition. Isoenzyme activity inhibits cytochrome P450 CYP1A1 and CYP1A1 or DMBA, causing non-reactive carcinogens [1,9]. Therefore, this study aims to determine the effect of *Elephantopus scaber* extract on breast cancer based on the immune system mechanism. It is supported by previous study, which stated that *E. scaber* had an ability as an anti-cancer agent [10]. This study used breast cancer mice model generated by DMBA and estrogen hormone induction.

MATERIAL AND METHOD

Twenty-seven female albino mice (BALB/c) with 4-5 weeks old were obtained from the Malang Murine Farm, Singosari. Mice acclimatized for seven days before experiments. Then, they were randomly divided into five groups, including a control group, DMBA-treated group, extract-treated group for week treatment, extract-treated group for 2 weeks of treatment, and extract-treated group for 3 weeks treatment. This research was approved by the institutional ethical committee of Brawijaya University, No.648-KEP-UB, 8th December 2016.

Extraction of *Tapak liman* (*Elephantopus scaber*)

Powder of *E. scaber* leaf obtained from Material Medica Batu, Malang. Powder of *Elephantopus scaber* macerated with ethanol 50% by comparison bulbs: ethanol 50% = 1:10 at room temperature during 5x24 hours, and stirred 3 times a day until all the components completely extracted. Thereafter, the ethanol extract filtered with a filter cloth and placed into a glass jar. The rest of the filtering material soaked again with ethanol 50% for 48 hours. Then, the material filtered by using filter cloth. The result of extraction evaporated to remove the ethanol content by using a water bath at 50°C and vacuum pump evaporator. Crude extracts were collected in the paste form, dark brown, and has a distinctive smell. Then, it placed in jar lid films and stored in a refrigerator at 4°C. Extract of *E. scaber* dose 50 $mg.kg^{-1}$ BW prepared for an experiment by dissolved it in warm water to make a stock solution.

Injection of DMBA and Estradiol

Five weeks old mice injected with DMBA (7,12-Dimethylbenz(α)antrasena) at the rate of 0.56 $mg.kg^{-1}$ BW. It was administered by subcutan (s.c) injection with a volume 3 $\mu L.g^{-1}$ BW. Furthermore, mice were injected with estradiol at the rate of 0.0504 $mg.kg^{-1}$ BW. The injection of DMBA and estradiol conducted weekly. Estradiol injection addressed to accelerate cancer growth.

Research Design

This study carried out by using five groups of mice consisting of negative control groups (K-), positive control (K+), and DMBA-estradiol-induced treatment groups then given extract of *E. scaber*. The treatment group consisted of three groups, namely the treatment group 1 week, which carried out with oral administration of extract E. scaber for a week. The second group is with oral administration of the extract of E. scaber for two weeks, and the third group for three weeks. Treatment was conducted by oral administration after mice injected with DMBA and estradiol for eight weeks and evaluated as positive breast cancer.

Lymphocyte Cell Isolation

Mice were sacrificed and dissected to isolate the spleen and liver organ. The obtained organ was washed and squeezed with PBS solution in a separate dish until all the cells become soluble. Then, the pellet suspension was filtered and put into a 15 mL polypropylene tube until the volume reached 10 mL. The suspension centrifuged at 2500 rpm at 10°C for 5 minutes. The supernatant discarded, and the pellet resuspended in 1 mL of PBS.

Preparation and Flowcytometry Analysis

The pellet suspension from each sample about 50 µL inserted into a sterile microtube containing 500 µL of PBS. The suspension was centrifuged with 2500 rpm at 10°C for 5 minutes. Extracellular staining carried out by adding 50 µL of monoclonal antibody (Fluorescein isothiocyanate (FITC) - conjugated CD4 anti-mouse rat (BiolegendTM), Phycoerythrin (PE) - Clocked anti-mouse CD8 rat (BiolegendTM), then incubated at 4°C for 20 minutes. Furthermore, it

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was added with 300 µL PBS and transferred to the Cuvet. The intracellular staining was conducted by adding 50 µL of cytofix-cytoferm (SIGMATM) to the pellet suspension and incubated at 4°C for 20 minutes. After incubation, 500 µL of washperm (SIGMATM) added and centrifuged with 2500 rpm at 10°C for 5 minutes. Pellets were collected and stained with intracellular antibodies (TNF-α anti-mouse rat (BiolegendTM) and PE - IFN-γ (BiolegendTM) anti-mouse conjugate) then incubated at 4°C for 20 minutes. Moreover, 300 µL PBS added and inserted into the cuvet of flow cytometry. In detail, the total number of cells was selected so that it could be detected by the antibody label. The obtained results were processed by BD cell quest ProTM software.

Statistical Analysis

The obtained data were analyzed by the CellQuest software and calculated by statistical analysis of ANOVA with P <0.05. The analysis data were conducted by using SPSS 16.0 for the Windows program. The data were generated in the form of a relative number of cells tested statistically by the normality test and variance homogeneity test. Then, the normal and homogeneous tests carried out for further analysis by using Two-way ANOVA with α = 0.05. Furthermore, it tested with the Tukey HSD test (High Significant Difference).

RESULT AND DISCUSSION

Analysis the Number of $CD4^+$

The $CD4^+$ T cells from spleen, which analyzed by using flowcytometry showed that the average length of the extract administration significantly affected each treatment group. The p-value <0.05 means that the average number of $CD4^+$ T cells in each treatment is significantly different. The interaction between treatment and duration of administration of extracts showed a p-value <0.05. It means that treatment and the duration of administration of extracts have an interaction.

The analysis results of $CD4^+$ T cells by using flowcytometry after DMBA and estradiol induction showed that there was a decrease in the positive control group (carcinogen induction) (Table 1). It occurred significantly (p <0.05) on a weekly basis. The cell number in the first, second, and the third week was 17.34%, 10.55%, 4.34%, respectively.

In the first week, the immune system recognized the presence of antigens so that the immune system in the body responded by producing effector cells such as $CD4^+$ T cells. Moreover, the low number of cells in the second and third weeks could be caused by the immune system in the body started to be recovered by itself or it could be explained that the administration of DMBA has no effect on mice.

It means that the body's response decreases or the body are in the homeostatic state. According to the previous study, the number of $CD4^+$ in T cells in breast cancer subjects was significantly decreased (p <0.05) [11]. In addition, the number of $CD4^+$ T cells in the final stage decreased as the previous stage. $CD4^+$ T cells play an important as an antitumor function by producing IFN-γ and TNF-α cells to activate cytotoxic T cells. Activated T cells will activate the cytotoxic T cells, macrophages, and NK cells to prevent the growth of tumors through the lysis process.

The relative number of $CD4^+$ in the DMBA-Estradiol induced cancer group had a lower number compared to the normal group. It could be caused by the cancer condition, which excreted of several cytokines such as TGF-beta. That cytokines are immunosuppressive cytokines types. It will promote suppression of effector T cells so that $CD4^+$ acts as an immunocompetent of IFN-γ cytokine producer and cytotoxic cells to destroy the tumor cells [3].

The herbal treatment for the cancer mice model can reduce the number of $CD4^+$ T cells. It presented by the relative number of $CD4^+$ cells in the first week to the third week of 7.23%, 2.54%, and 5.59%, respectively (Fig. 1). The *E. scaber* extract administration for three weeks did not show a significant result compared to the extract administration for two weeks. Therefore, the administration of extract for three weeks did not affect $CD4^+$ T cells.

Table 1. Relative number of $CD4^+$ and $CD8^+$ T cells from spleen organs

	CD4+ (%)			CD8+ (%)		
	1st week	2nd week	3rd week	1st week	2nd week	3rd week
K-	20.75^{de}	21.91^{e}	21.21^{de}	9.96^{b}	13.11^{c}	16.68^{d}
K+	17.34^{d}	10.55^{c}	4.34^{ab}	17.03^{d}	9.76^{b}	8.5^{b}
P	7.23^{bc}	2.54^{a}	5.59^{ab}	9.31^{b}	1.3^{a}	10.19^{b}

Notes: K- = healthy mice, K+ = induced DMBA and Estradiol mice, P = Induced DMBA and Estradiol treated and administration of *E. scaber* extract 50 $mg.kg^{-1}$ BW.

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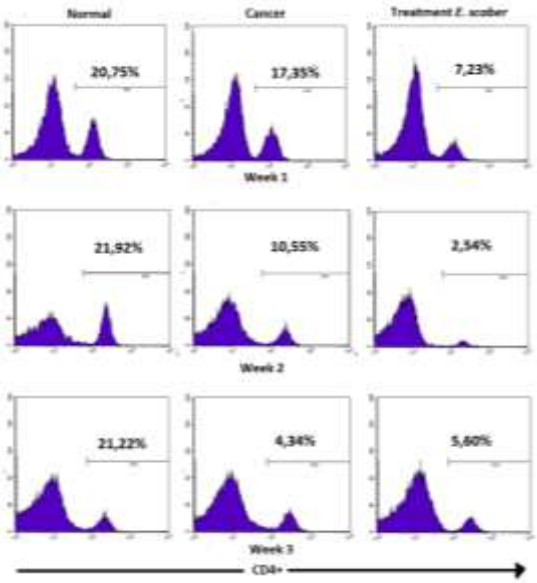

Figure 1. Percentage of the relative number of $CD4^+$ T cell at the spleen organ.

Elephantopus scaber extract contains several compounds such as flavonoids, alkaloid saponins, tannins, phenols, steroids, isodeoxyelephantopin, terpenoids, and deoxyelephantopin [7]. Cheeke [12] stated that saponin and flavonoid compounds had a function as natural immunomodulators. Saponins and flavonoids can enhance the immune responses, especially for increasing the number of immunocompetent cells such as $CD4^+$ T cells.

However, this is not comparable with the obtained results in breast cancer case after administration of DMBA and estradiol. It showed that the compounds of *E. scaber* extract have not been able to increase the number of $CD4^+$ T cells in mice after DMBA and estradiol administration. However, according to the previous study, a high dose of *E. Scaber* leaf extract can reduce the number of $CD4^+$ T cells [13].

The importance of CD4 T cells as antitumor immunity can be emphasized in several aspects. First, the initial idea that CD4 T cells provide assistance to induce and maintain a specific response to CD8 cytotoxic T cells. Second, CD4 T cells can develop and maintain CD8 memory T cells. Third, the tumor rejected by cytotoxic effects of CD4 T cells, regulation of MHC molecules, antiangiogenesis, and tumor lysis induction [14]. So in this case, the tumor formed rapidly than the immune system in the body.

Analysis the Number of CD8+ T Cells

The analysis results of CD8 T cell from spleen which analyzed by using flowcytometry showed that the average length of administration of the extract significantly affected each treatment group. In addition, it appears that p-value (<0.05) means that the average number of CD8 T cells in each treatment was significantly different. The interaction between treatment and duration of administration of extracts showed p<0.05, which means that there was an interaction.

Based on the $CD8^+$ T cell analysis from spleen (Table 1), it showed that the DMBA-induced group of mice and estradiol (positive control) had a relative cell number of 17.03% in the first week, 9.76% on the second week, and 8.50% on the third week. The relative number of $CD8^+$ T cells increased on the first week compared to healthy mice (negative controls). It showed a significant increase compared to healthy mice or negative controls.

However, the relative number of $CD8^+$ T cells at weeks 2 and 3 decreased. It means that $CD8^+$ T cells, which had a function as cytotoxic T cells, were formed in the first week to fight tumor cells and regulate the cellular and humoral immune system, such as activating Th2 lymphocytes by secreting cytokines that induce B lymphocytes to become plasma cells [3]. The decreased number of $CD8^+$ T cells at week 3 demonstrated that there was a process of homeostasis in the body in neutralizing the presence of foreign objects that enter the body.

$CD8^+$ T cells have special functions in lytic functions, and they also has the ability to express MHC class I. Therefore, it is believed that $CD8^+$ T cells are the main effector cells which responsible for eliminating tumor cells. However, tumor-reactive $CD4^+$ T cells can develop cytotoxic activity and mediate tumor rejection through the introduction of class II MHC antigens [15,16]. Cells that contain tumor antigens will bind to the MHC I. Then, it will form a complex through TCR (T Cell Receptor) from CD8 T cells (cytotoxic) so that CD8 T cells are activated and play a role in cell lysis [8,17]. According to the previous study, the number of CD8 T cells in breast cancer subjects was also significantly decreased ($p < 0.05$). In addition, the number of CD8 T cells in the final stage decreased compared to the previous stage.

The mice group induced by DMBA and estradiol also treated with *E. scaber* extract. The result showed a significant decrease ($p < 0.05$) compared to the cancer group (positive control) in the first week and the second week. Previous studies exhibited that the effects of the *Elephantopus scaber* at doses of 0.5 $g.g^{-1}$ BW and

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1.0 $g.g^{-1}$ BW could increase the CD8 T cells number, but decrease the number of CD4 and CD8 T cells at a dose of 2.0 $g.g^{-1}$ BW. The lower doses could stimulate the proliferation of T cells, while at high doses, it could prevent the T cell proliferation [19].

The number of CD4 and CD8 T cells in this study showed that the *E. scaber* extract played a role as immunosuppressants of $CD4^+$ and $CD8^+$ T cells in the second week. It is due to the content of lupeol and flavonoids, which act as an anti-inflammatory agent. Previous research has also shown that the effects of herbs can stimulate or otherwise suppress immunity [19,20]. The low number of $CD8^+$ T cells in mice after administrated by *E. scaber* extract probably caused by the content of isodeoxyelephantopin on *Tapak Liman* leaves [8]. It is known to increase the number of regulatory T cells for suppressing excessive immuno-competent cells inside the body.

The number of $CD4^+$ T cells and $CD8^+$ T cells decreased due to the activation of $CD4^+$ cells which developed into Th1 and Th2 cells [13]. In addition, the response of $CD8^+$ T cells will increase the pro-inflammatory cytokines released by $CD4^+$ T cells. Furthermore, it also has a function to activate the immunocompetent cells such as IFNγ, which play a role in increasing the expression of MHC I so that it can be recognized by $CD8^+$ T cells. Moreover, the $CD8^+$ T cells naive can be activated into T cells effector. However, the decreased number of $CD4^+$ T cells will cause T cells naive to be unable to differentiate into effector $CD8^+$ T cells optimally.

CD8 T cells are the essential mediator in the body's immunity system to several pathogens, including viruses, intracellular parasites, and bacteria. It also has a function as immune protection against cancer. The optimal induction response of CD8 T cells is very important for our defense against infective disease or cancer. Cytotoxic T lymphocytes (CTLs) play a major role in the rejection of immunogenic tumors. Cellular immune responses are the process of presenting protein antigen-presenting cells (APCs) and current antigenic peptide fragments through the MHC II complex to CD4 T cells. Activated CD4 cells will help the effector cells through cytokine production [17,18].

The results of the number of CD4 and CD8 T cells have almost the same number in cancer cases. The total number of CD4 T cells in the first week was about 17.34%, while CD8 T cells were 17.03%. The CD4 cell number in week 2 was 10.55%, while in CD8, it was 9.76%. Whilst, the CD4 number at week 3 was 4.34%, and the CD8 cells were 8.50% (Fig. 2).

The CD4 T cells play an important role in facilitating the initial activation and development of CD8 T cells. During the CD8 phase priming, the T cells are activating CD4 T cells so that it can help to activate the CDL CTL. The CD4 T cells regulate the antitumor CDL CTL response through direct cell interaction and IL-2 stimulation. CD4 T cells can directly help activate CD8 cells through CD40-CD154 interactions [21,22,23].

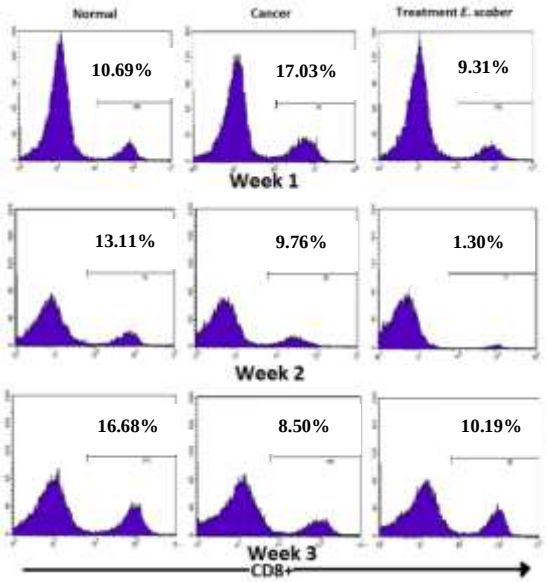

Figure 2. Percentage of the relative number of $CD8^+$ T cell at the spleen organ.

Analysis the Number of $CD4^+TNF\text{-}\alpha^+$ T Cells

TNF-α is a pleiotropic cytokine produced by various nucleated cells in the body. TNF-α is a pro-inflammatory agent that will regulate macrophages to respond to trauma, infection, and cell stress, such as tumors. However, the main function of TNF-α is as a mediator in producing other proinflammatory cytokines such as IL-1 and IL-6 [24].

Based on the results of this study, the relative number of cells in the normal mice group (K-) was lower than the DMBA and estradiol induction group. However, the treatment group treated by *E. scaber* extract has a significant difference as compared to the normal group (K-). The group that had been induced by DMBA and estradiol (K+) had a total number of 3.06%, and it was significantly different from the normal group 2.09%. It means that the immune system performed self-recovery after exposure to the DMBA and estradiol.

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Table 2. Relative number of $CD4^+TNFa^+$ and $CD4^+IFNg^+$ T cells from spleen organs (%)

	CD4+TNFa+ (%)			CD4+IFNγ+ (%)		
	1st week	2nd week	3rd week	1st week	2nd week	3rd week
K-	1.58^{a}	3.34^{bc}	1.35^{a}	2.67^{ab}	4.03^{bc}	4.62^{bc}
K+	2.77^{ab}	1.19^{a}	5.19^{de}	5.99^{c}	1.126^{a}	2.20^{ab}
P	6.69^{e}	6.13^{de}	4.59^{cd}	15.15^{e}	12.35^{d}	2.54^{ab}

Notes: K- = healthy mice, K+ = induced DMBA and Estradiol mice, P = Induced DMBA and Estradiol treated and administration of *E. scaber* extract 50 $mg.kg^{-1}$ BW.

The treatment of *E.scaber* extract in mice exposed to DMBA and estradiol showed a significant difference ($p < 0.05$) about 5.81% compared to the normal group and positive controls (exposure to DMBA and estradiol). This high number means that the active ingredient in *E. scaber* extract can improve the immune system to actively protect the body and improve the tissue after DMBA exposure [25].

NF-α is cytotoxic for many types of tumor cells, and it is capable for triggering the cascade signal transduction, such as in the process of apoptosis, stimulating NF-kB, and the activation of p38 MAPK, ERK, JNK. The TNF-α attachment in TNFR1 will activate the TNFR-associated death domain (TRADD) as adapter proteins. This protein will interact with the cytopathic death domain on TNFR1 via death domain and activate the apoptosis process through the caspase pathway [26,27]. Therefore, we observed that TNF-α has a strong relationship with the level of tumors.

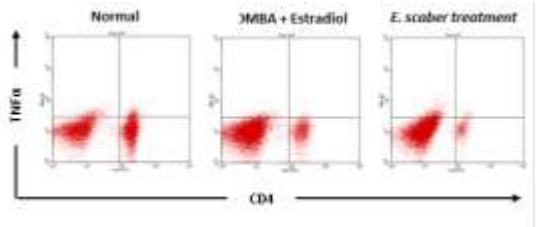

Figure 3. The relative number of $CD4^+$ TNF-α^+ T cell at the spleen organ

Analysis the Number of $CD4^+IFN\text{-}\gamma^+$ T Cells

Proinflammatory cytokines such as IFN-γ is a cytokine which expressed and synthesized by NK cells. Interferon also plays a role in increasing and inhibiting cell function. The inhibition function to reduce the normal cell growth and neoplastic cells. The function of enhancement addressed to kill bacteria by activating macrophages. The cytokines will activate macrophages in phagocytizing pathogens effectively. Also, the IFN-γ cytokine will stimulate the response of some antibodies. IFN-γ cytokines play a role in pro-inflammation and induction of type I immunity. The presence of these cytokines used to measure the immune system's work [28,29].

Based on the experiment results, it showed that *E. Scaber* extract was significantly increased the relative number of $CD4^+IFN\text{-}\gamma^+$ cells ($p < 0.05$) on the first week (15.16%) and second (12.35%) (Table 2). Healthy mice have a relative number of $CD4^+IFN\text{-}\gamma^+$ cells that are not significantly different. It means that the relative number of $CD4^+IFN\text{-}\gamma^+$ cells at week 1, week 2, and week 3 had the same results. When compared to DMBA and estradiol-induced mice, the healthy mice had a significant difference in the first week (5.99%), second week (1.13%), and the third week (2.20%). However, in the first week, DMBA and estradiol-induced mice had a significantly higher number of cells ($p < 0.05$) as compared to the second and third weeks. It demonstrated the self-recovery in mice after being injected with DMBA and estradiol on the first week.

Meanwhile, in the second and third weeks, there was a decreased number of $CD4^+IFN\text{-}\gamma^+$ cells, which showed that there was no defense to inhibit the DMBA and estradiol carcinogens. The administration of *E. Scaber* extract also showed a significant decrease in every week. It means that in the first week, the number of IFN-γ cytokines has increased to activate macrophages. Whereas, in the second and third weeks, there were a decreased IFN-γ cytokines. It indicated that the *E. scaber* extract has not been able to maintain the amount of IFN-γ because the carcinogen substance in the body of the mice increased.

However, as compared to the positive control group of mice exposed to DMBA and estradiol in the second week, it showed a significant increase (12.35%). But, in the third week between the administration of DMBA and estradiol (2.20%) and the administration of the extract, it did not show a significant difference (2.54%) (Fig. 4). It explained that the carcinogen level in the mice's body could not be controlled by the immune system.

Under normal conditions, the body does not specifically respond to carcinogen agents so that the number of IFN-γ cytokines decreases. However, in the exposure to the DMBA and estrogen carcinogens, the body had self-recovery ability to fight the carcinogen. Treatment of *E.*

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Scaber extract can increase the amount of $CD4^{+}IFN\text{-}\gamma^{+}$ to inhibit the carcinogenesis process in mice after induction of DMBA and estradiol [28]. IFN-γ activity is including the activation of macrophage cells and CTL in anti-tumors, increasing NK cell function as potent effector cells in lysis of cancer cells, and controlling the regulation of B lymphocytes in the immune response. Activation of macrophages by IFN-γ will secrete the activator cytokines, namely IFN-γ. This cytokine will activate the CTL cells and NK cells to kill cancer cells through the Fas-ligand pathway then activate FADD. The FADD activity will trigger the cascade activity to promote DNAse for the apoptosis process [30].

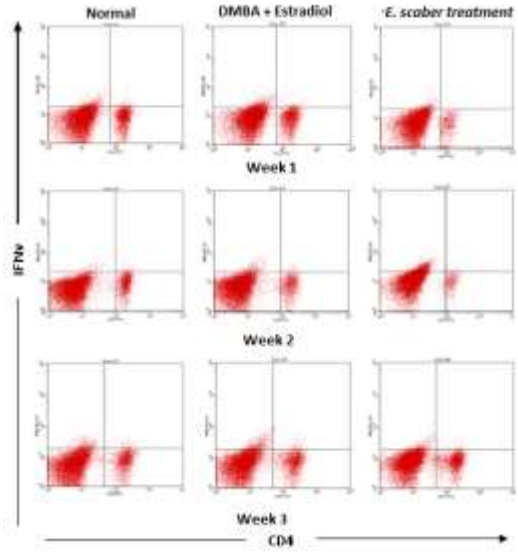

Figure 4. Percentage of relative number of $CD4^{+}$ $IFN\text{-}\gamma^{+}$ T cell at the spleen organ

CONCLUSION

The results showed that the induction of DMBA and Estradiol carcinogens in mice had an effect of fur loss on mice. It also has an effect on decreasing the immune system through CD4 and CD8 T cells. However, the *E. scaber* extract can increase the number of proinflammatory cytokines of TNF-α. Moreover, it also promoted the number of proinflammatory cytokines of $CD4^{+}$ $IFN\text{-}\gamma^{+}$ in the first week and decreased the number of $CD4^{+}IFN\text{-}\gamma^{+}$ in the second and third weeks. Then, it increased the number of $CD4^{+}$ $TNF\text{-}\alpha^{+}$. The treatment of *E. scaber* extract could increase the number of $CD4^{+}IFN\text{-}\gamma^{+}$ in the first and second week. In brief, the treatment of *E.scaber* extract can restore the homeostasis of the immune system through the number of CD4 and CD8 T cells in the first and second week.

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Effect of Pb and Cd Elicitors on Growth and Content of Vetiver Oil Adventitious Root *In Vitro* of Vetiver (*Vetiveria zizanioides* L. Nash)

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Abstract

This research aimed to evaluate the effect of Pb and Cd elicitors on root growth and essential oil content of vetiver oil on the root culture of vetiver (*Vetiveria zizanioides* L. Nash). Roots were induced by culturing of the shoot on MS media supplemented with 0.1 mg.L^{-1} kinetin and 7 mg.L^{-1} NAA. Elicitation was done by culturing roots on MS media with 0.1 mg.L^{-1} kinetin and 7 mg.L^{-1} NAA + 0.1 mM Pb or Cd. Roots then observed for fresh weight, dry weight, number, and root length, and vetiver oil contents were analyzed using GC-MS. The addition of Pb and Cd heavy metal elicitor affected the formation and the content of vetiver oil compounds of root culture. Pb elicitor increased the number of roots, while Cd elicitor increased root length. However, the addition of Pb and Cd elicitor on culture media decreased the fresh weight and root dry weight. Fifty eight vetiver oil compounds in adventitious roots of vetiver plants were identified. The main compounds of vetiver oil in roots culture were Khusimone (6.94%), Khusimol (6.05%), Khusimene (4.85%), α Vetivone (3.70%), β Vetivone (3, 53%), Vetiverol (3.22%), Prezizaene (2.35%), and Zizaene (1.91%). Elicitation with Pb and Cd increased the composition of the main compounds of vetiver oil. Cd elicitor increased the composition of the main compound vetiver oil higher than the Pb elicitor.

Keywords: adventives root, elicitor, in vitro, Pb and Cd, vetiver oil

INTRODUCTION

Vetiver plant (*Vetiveria zizanoides*) is an essential oil-producing plant and widely used for industrial needs as a basic ingredient of the perfume, cosmetics, and soap [1]. The main compounds composing vetiver essential oil are sesquiterpenic hydrocarbons such as cadenene, clovene, aromadendrine, and carbon compounds such as vetivone and khusimone which are the main compounds producing odor, as well as characteristic compounds of vetiver oil [2].

Essential oil production has reached 250 tons per year on a global scale. The needs of essential oils have not been fulfilled due to production that were dominantly done using conventional technology. The use of conventional technology produces oils with quality that does not meet consumer needs. One way to increase the production and quality of essential oils is by using the culture method. Tissue culture techniques can be carried out through callus culture, cell suspension, and root culture (adventitious and root tip) [3]. Large-scale production of ginsenoside successfully carried out through ginseng adventitious plant root culture [4].

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Culture techniques to increase secondary metabolite production can be done using elicitor [4]. Elicitors are compounds which stimulate physiological disorders in plants that can be used to increase the synthesis of secondary metabolites. Previous study have stated that the addition of Pb metal elicitor in vetiver plant increased the activity of enzymes that play a role in the synthesis of vetiverol [5]. Cd metal elicitor in *Datura stramonium* root culture increased the production of sesquiterpenoid compounds [6]. Therefore, this study aims to determine the effect of Pb and Cd elicitor on growth and components of vetiver oil on the vetiver plant by in vitro.

MATERIALS AND METHOD

Induction of Adventitious Root *In vitro* of Vetiver

Adventitious roots induced by culturing shoots (±2 cm) on MS media with the addition of 0.1 mg.L^{-1} Kinetin + 7 mg.L^{-1} NAA. Root cultures incubated with 600 lux light at temperature of 25-26°C for four weeks. Formed roots multiplied on liquid MS media with the same type of growth regulator.

Elicitation of Root *In vitro* with Pb and Cd

Roots (0.1 g) were cultured in liquid MS media + 0.1 mg.L^{-1} Kinetin + 7 mg.L^{-1} NAA + 0.1 mM Pb or Cd elicitor. The medium without any addition of an elicitor was used as a control. Cultures incubated with 600 lux light at temperature of

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25-26°C for four weeks. Each treatment was repeated five times. The culture was evaluated for the growth of roots (fresh and dry weight, number and length, of the root). Vetiver oil components analyzed by the GC-MS method.

Analysis of *Vetiver oil* compound content of Root using *Gas Chromatography-Mass Spectrometry* (GC-MS)

Roots were pulverized and weighed at 0.1 g. The samples were extracted three times with hexane in a ratio of 1:5 (sample: hexane) and stirred until homogeneous. Samples were incubated overnight at cold temperatures. The solution filtered with an Erlenmeyer vacuum filter, so that filtrate and residue were obtained. The filtrate were combined and evaporated using a rotary evaporator to separate hexane solvents so that an extract obtained. The extracted solution was centrifuged at 5000 rpm for 20 minutes to obtain the supernatant. The supernatant was then diluted using hexane. The supernatant was vortexed and filtered with 0.45 µm membrane cellulose acetate. Vetiver oil compound analysis was carried out by the Gas Chromatography-Mass Spectrometry (GC-MS) Shimazu QP 2010 SE method. The GC-MS apparatuses consist of 15 Kpa helium as the carrier gas, ZB-AAA column type (10 m x 0.25 mm), column temperature of 110-320°C, and injector temperature of 200°C.

RESULT AND DISCUSSION

Effect of Pb and Cd Elicitors on The Growth of Root *In vitro*

Initial growth responses of root explants were indicated by shoot formation after one week of culture. Formation and growth of roots occur after two weeks of culture. The roots of the control media were yellowish-white (Fig. 1A), whereas on the culture media with the addition of Pb elicitor were yellowish-white, and green (Fig. 1B). The root of the culture medium with the addition of Cd elicitor had a color variation were yellowish-white, green, and several others were green with an orange-colored end (Fig. 1C).

Figure 1. *In vitro* roots of elicitation treatment. **Description:** (A) Control, (B) Pb elicitor, (C) Cd Elicitor.

Elicitation of heavy metals in root culture affected fresh weight and roots dry weight. The addition of Pb and Cd elicitor on culture media significantly reduced fresh weight and dry weight compared to the control. The fresh and dry weight of roots in the control media were 144 mg and 12 mg, while the fresh and dry weight of the media with the addition of elicitor were 118 mg and 10 mg with Pb elicitor and 134 mg and 11 mg with Cd elicitor. The decreasing of roots fresh and dry weight in media with the addition of Pb elicitor was higher than that of the Cd elicitor (Fig. 2).

The addition of Pb and Cd elicitor on culture media affected the number and length of the roots. The addition of the Pb elicitor inhibited the growth of root length, but did not significantly affect the number of roots, although there was an increase in the number of roots. The number of roots on the addition of Pb and Cd elicitor was not significantly different compared to the control. However, in the Pb elicitation media, the number of roots produced was significantly higher than the number of roots on the Cd elicitation medium. The addition of Pb on the elicitation media inhibited the growth of root length significantly compared to the control. The roots on the Cd elicitation medium were significantly longer compared to the roots on control and Pb elicitation treatment (Fig. 3).

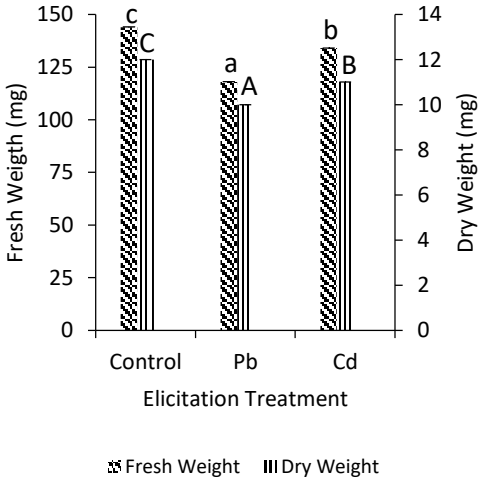

Figure 2. Effect of Pb and Cd licitors on the fresh weight and dry weight of roots *in vitro* of vetiver. **Note:** the same letter on each bar showed not significant difference according to Duncan test (α=0.05).

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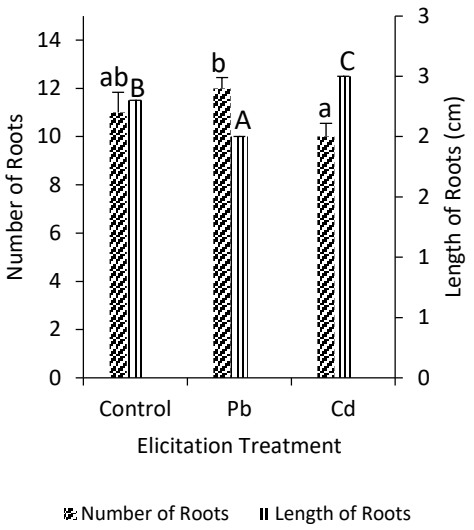

Figure 3. Effect of Pb and Cd elicitors on the number and length of roots *in vitro* of vetiver. **Note:** the same letter on each bar showed not significant difference according to Duncan test (α=0.05).

Elicitor divided into two types, namely biotic and abiotic. Heavy metal was one of the effective abiotic elicitors and had often been used to stimulate the production of secondary metabolites in tissue culture [7]. The use of $CdCl_2$ elicitor increased roots growth and formation in *Brugmansua candida* roots culture [8]. The low concentration of Cd elicitor (5.0 µM) gave the best response to increase the number of roots, and an increase in secondary metabolite compounds of andrographolide in *Andrographis paniculata* plants [9].

Effect of Pb and CD Metal Elicitors on the Component of *Vetiver Oil* compound of *Roots*

The results chromatogram in roots culture on control medium showed 58 compounds (Fig. 4A), but on medium with Pb elicitor, 52 compounds were identified (Fig. 4B), and on medium with Cd elicitor, 50 compounds were identified (Fig. 4C). The retention time for vetiver oil compounds in each treatment was the same, but the percentage composition of each compound was different for each treatment.

The main compounds of vetiver oil were identified both in control roots and treated roots namely khusimone, khusimol, khusimene, α vetivone, β vetivone, vetiverol, prezizaene, and zizaene (Fig. 4). The identified components of the main vetiver oil appear at different times. Zizaene was the first major compound of vetiver oil identified at 24.7 minutes, followed by prezizaene at 25 minutes, khusimene in minute 25.1, khusimone in minutes 29.9, vetiverol at minute 32, β vetivone in minutes to 35.8 and α Vetivone in minutes 36.5 (Table 1).

The composition of main compounds of vetiver oil in the control treatment were zizaene 1.81%, prezizaene 2.23%, khusimene 4.59%, khusimone 6.58%, vetiverol 3.05%, khusimol 5.73%, β vetivone 3.35% and α vetivone 3.50%. Khusimone was the compound that has been identified as having the highest composition, while zizaene has the lowest composition compared to the other main compounds. The treatment of Pb and Cd elicitation caused an increase in the composition of the main compounds of vetiver oil in roots culture. Cd elicitor was able to increase the composition of the main compound in vetiver oil in root culture higher than Pb heavy metal elicitor (Table 1 & Fig. 5).

In the elicitation treatment, fewer vetiver oil compounds were identified, 52 of which were elicited with Pb, and 50 in the results of elicitation with Cd. This amount is less than in controls (58 compounds). Among these compounds, 6 compounds were not detected in the results of Pb elicitation compared to controls, namely Isoeugenol, δ Selinene, Elemol, β Atlantol, 10 Epi γ eudesmol, and Anhydro β rotunol.

The elicitation treatment with Cd caused some compounds not to be detected compared to controls, namely Myrcene, Isoeugenol, δ Cellinene, Agarospirol, 10 Epi γ eudesmol, Cubenol, and Anhydro β rotunol. Some of the compounds were not identified in the elicitation treatment with Pb and Cd. It possibly caused by the elicitation properties used, namely heavy metals, which cause toxicity resulting in inhibition of the synthesis process.

The main components of vetiver oil consist of sesquiterpenes, sesquiterpenol, and sesquiterpenon such as benzoate acid, vetiverol, furfurol, α and β vetivone, vetivene, and vetivenyl vetivenate [10]. The type of elicitor influenced the content of secondary metabolites produced. Also, the concentration of elicitor added to culture media also affected the content of secondary metabolites in a sample. Each elicitor had the ability to increase secondary metabolites, but the concentration of each component was not the same. The optimum concentration of an elicitor was able to increase the content of secondary metabolites in a sample [11].

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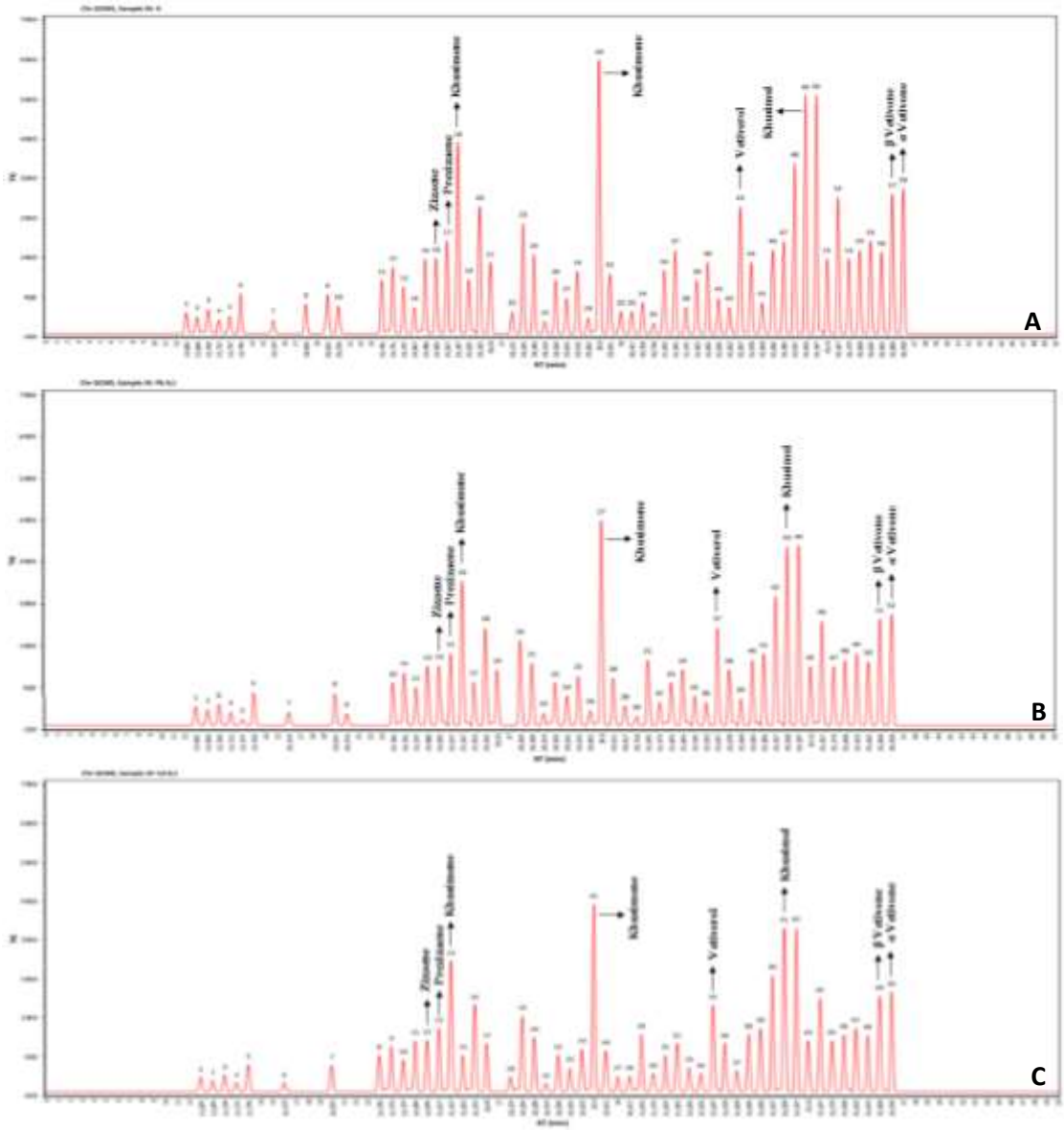

Figure 4. Chromatogram of GC-MS *vetiver oil* of roots *in vitro* of vetiver with elicitation treatment (A) Control, (B) Pb, (C) Cd.

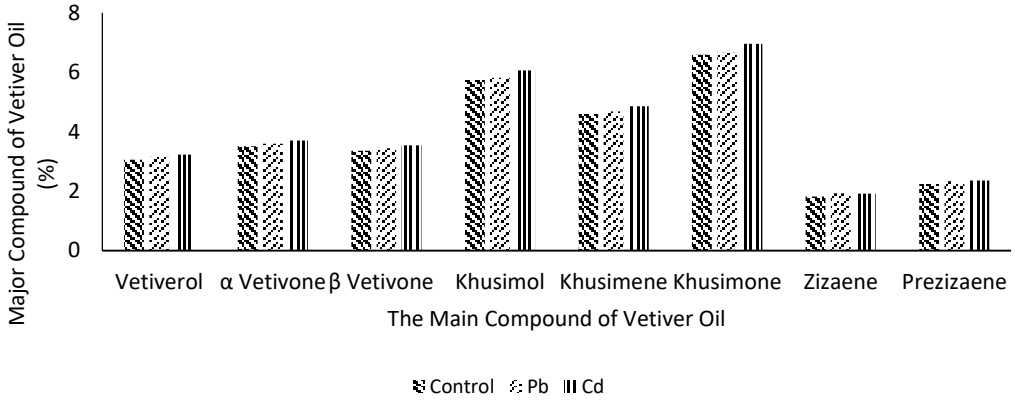

Figure 5. Effect of Pb and Cd Elicitors on *Vetiver Oil* Main Compound of Adventitious Root of Vetiver

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Table 1. Percentage of chemical compound of *vetiver oil* from adventives roots of *in vitro* vetiver plants result of elicitation with metal

No	Compounds Name	Formula Structure	Retention time (minutes)	Percentage/Content of *Vetiver Oil* Compounds on Elicitation Treatment		
				Control	Pb	Cd
1	4 Vinylphenol	C_8H_8O	13.682	0.49679	0.61116	0.52442
2	α Thujene	$C_{10}H_{16}$	13.694	0.39244	0.50764	0.41427
3	α Pinene	$C_{10}H_{16}$	13.704	0.57125	0.68504	0.60303
4	Camphene	$C_{10}H_{16}$	13.722	0.31486	0.43068	0.33238
5	Myrcene	$C_{10}H_{16}$	13.737	0.41916	0.19530	-
6	Limonene	$C_{10}H_{16}$	13.746	0.94590	1.05673	0.99851
7	Linalool	$C_{10}H_{18}O$	16.119	0.31380	0.42962	0.33126
8	Isoeugenol	$C_{10}H_{12}O_2$	18.636	0.69722	-	-
9	Khusitene	$C_{14}H_{22}$	20.015	0.91834	1.02939	0.96943
10	Cadalene	$C_{15}H_{18}$	20.376	0.65955	0.38959	-
11	α Funebrene	$C_{15}H_{24}$	24.785	1.27265	1.38090	1.34344
12	Zizanene	$C_{15}H_{24}$	24.791	1.58895	1.69470	1.67734
13	Acora 4,9 diene	$C_{15}H_{24}$	24.797	1.11669	1.22617	1.17880
14	δ Selinene	$C_{15}H_{24}$	24.807	0.63073	-	-
15	Valencene	$C_{15}H_{24}$	24.986	1.79981	1.90390	1.89993
16	**Zizaene**	$C_{15}H_{24}$	24.995	1.81533	**1.91930**	**1.91631**
17	**Prezizaene**	$C_{15}H_{24}$	25.017	2.23138	**2.33206**	**2.35550**
18	**Khusimene**	$C_{15}H_{24}$	25.167	4.59593	**4.67794**	**4.85158**
19	γ Gurjunene	$C_{15}H_{24}$	25.925	1.28152	1.38970	1.35281
20	β Vetispirene	$C_{15}H_{22}$	26.242	3.05781	3.15197	3.22791
21	γ Amorphene	$C_{15}H_{24}$	26.35	1.69342	1.79835	1.78761
22	Elemol	$C_{15}H_{26}O$	28.125	0.51115	-	0.53958
23	β Vetivenene	$C_{15}H_{22}$	28.283	2.65284	2.75020	2.80041
24	α Vetispirene	$C_{15}H_{22}$	28.289	1.90469	2.00795	2.01064
25	β Bisabolol	$C_{15}H_{26}O$	28.314	0.228624	0.40228	0.302216
26	Maaliol	$C_{15}H_{26}O$	28.583	1.27365	1.38190	1.34450
27	Juniper camphor	$C_{15}H_{26}O$	29.015	0.83244	0.94417	0.87875
28	Viridiflorol	$C_{15}H_{26}O$	29.525	1.48340	1.58999	1.56591
29	Agarospirol	$C_{15}H_{26}O$	29.662	0.37137	0.48673	-
30	**Khusimone**	$C_{14}H_{20}O$	29.9	6.58222	**6,64856**	**6.94837**
31	Cadinane	$C_{15}H_{26}$	29.911	1.42490	1.53195	1.50416
32	β Atlantol	$C_{15}H_{24}O$	30.000	0.52563	-	0.55486
33	Junenol	$C_{15}H_{26}O$	30.417	0.52562	0.63976	0.55485
34	10 Epi γ eudesmol	$C_{15}H_{26}O$	30.708	0.73726	-	-
35	Cubenol	$C_{15}H_{26}O$	30.758	0.24404	0.29603	-
36	Anhydro β rotunol	$C_{15}H_{20}O$	31.005	1.51022	-	-
37	1,7 Di epi α cedrenal	$C_{15}H_{22}O$	31.042	2.00915	2.11159	2.12092
38	α Cadionol	$C_{15}H_{26}O$	31.375	0.63197	0.74528	0.66713
39	Mustakone	$C_{15}H_{22}O$	31.825	1.27257	1.38082	1.34336
40	Zizanal	$C_{15}H_{22}O$	31.842	1.69447	1.79939	1.78873
41	Caryophyllene oxide	$C_{15}H_{24}O$	32.035	0.83304	0.94476	0.87938
42	Epikhusinol	$C_{15}H_{24}O$	32.053	0.63102	0.74434	0.66613
43	**Vetiverol**	$C_{15}H_{24}O$	32.067	3.05302	**3.14722**	**3.22285**
44	Zizanol	$C_{15}H_{24}O$	32.078	1.69510	1.80001	1.78939
45	Junicedranol	$C_{15}H_{26}O$	32.658	0.73727	0.84975	0.77828
46	Nootkatol	$C_{15}H_{24}O$	33.083	2.00900	2.11143	2.12075
47	Vetiselinenol	$C_{15}H_{24}O$	33.383	2.22073	2.32150	2.34427
48	Z β curcumin 12 ol	$C_{15}H_{24}O$	33.567	4.10643	4.19232	4.33486
49	**Khusimol**	$C_{15}H_{24}O$	34.058	5.73647	**5.80949**	**6.05558**
50	Khusol	$C_{15}H_{24}O$	34.187	5.75563	5.82849	6.07580
51	Isovalencenol	$C_{15}H_{24}O$	35.15	1.80051	1.90459	1.90066
52	Spirovetiva 3,7(11) dien 12 ol	$C_{14}H_{22}O_2$	35.267	3.28391	3.37628	3.46658
53	Allo khusiol	$C_{15}H_{26}O$	35.279	1.79958	1.90367	1.89968
54	Nootkatone	$C_{15}H_{22}O$	35.608	2.00808	2.11053	2.11978
55	Isokhusenic acid	$C_{15}H_{22}O_2$	35.633	2.22467	2.32541	2.34842
56	Zizanoic acid	$C_{15}H_{22}O_2$	35.642	1.96217	2.06498	2.07132
57	**β Vetivone**	$C_{15}H_{22}O$	35.858	3.35089	**3.44273**	**3.53729**
58	**α Vetivone**	$C_{15}H_{22}O$	36.558	3.50513	**3.59576**	**3.70011**

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Increased secondary metabolites with elicitor occur through activation of secondary pathways in response to stress, which results in a metabolic process that increases the activity of enzymes involved in the biosynthesis of secondary metabolites [12]. The addition of the Pb elicitor increases the component of Vetiverol compounds, higher than the control. Whereas, the addition of Cd activates the enzyme activity that plays a role in the synthesis of alpha sinensal and alpha amorphene in vetiver callus culture [5].

The addition of the Cd^{2+} elicitor gave a positive response to produce the production of alkaloids in the *Beta vulgaris* plant. The addition of Cd elicitor with a concentration of 1.0 mM gave the best response in increasing the production of secondary metabolites A and C inophyllums, as well as calophyllolide, whereas in callus suspension culture, increased the production of B and P inophyllums [13]. The addition of $CdCl_2$ with a concentration of 1 mM on culture media was able to effectively increase the production of andrographolide in *Andrographis paniculata* cell suppression culture by 4.14 times higher than the control [14]. Addition of 0.005 $mg.L^{-1}$ CdCl2 in *Rubia tinctorum* L. callus culture increased the flavonoid content of 57-64% compared to controls [15].

CONCLUSION

The addition of Pb and Cd heavy metal elicitor affected the formation and the content of vetiver oil compounds of root culture. Pb elicitor increased the number of roots, while Cd elicitor increased root length. However, the addition of Pb and Cd elicitor on culture media decreased the fresh weight and root dry weight. Fifty-eight vetiver oil compounds in the adventitious roots of vetiver plants were identified. The main compounds of vetiver oil in roots culture were Khusimone (6.94%), Khusimol (6.05%), Khusimene (4.85%), α Vetivone (3.70%), β Vetivone (3, 53%), Vetiverol (3.22%), Prezizaene (2.35%), and Zizaene (1.91%). The Cd elicitor increased the composition of the main compound in vetiver oil, that higher than the Pb elicitor.

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CFGWC-PSO in Analyzing Factors Affecting the Spread of Dengue Fever in East Java Province

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Abstract

Fuzzy Geographically Weighted Clustering-Particle Swarm Optimization using Context Based Clustering (CFGWC-PSO) has been developed to clustering in factors influencing the spread of dengue fever in East Java Province. CFGWC-PSO method can overcome slow computing time problems in terms of iterations, and produce accurate data partition with stable. In this research, CFGWC-PSO applied to 11 variables from data on the causes of the spread of dengue fever in East Java Province in 2017. CFGWC-PSO using the FCM method to determine the context variable. Processing used the results of clustering with 2 clusters until 5 clusters. From the three validation index that used to find out the right number of clustering, two clusters gave better clustering results. CFGWC-PSO shows that all districts/cities in cluster 2 become dengue fever endemic areas that need to be considered by the East Java Provincial Government.

Keywords: Context-Based Clustering, dengue hemorrhagic fever, Fuzzy Geographically Weighted Clustering-Particle Swarm Optimization.

INTRODUCTION

Dengue Haemorrhagic Fever (DHF) is an important health problem in Indonesia. It was first known in 1979 in Cairo, and in the same year it also happened in Asia, is Jakarta, which was still called Batavia. But actually, dengue fever in Indonesia was first discovered in the city of Surabaya in 1968. As many as 58 people were infected, and 24 of them died. Since then, this disease has spread throughout Indonesia [1].

DHF is a problem that is routinely in every rainy season and after the rainy season. DHF transmitted through the bite of the *Aedes aegypti* and *A. albopictus* mosquitoes that carry the dengue virus. Vector control always used to cut off the chain of transmission in preventing DHF. It is because there is no vaccine to prevent dengue virus. DHF vector is actually easy to control. However, because the vector spread widely, the success of the control requires total coverage (covering the entire area) for the mosquitoes cannot reproduce again.

Factors related to the increase in dengue and outbreak incidence in East Java that is difficult to control were based on the East Java Health Office [2]. It is related with population density, population mobility, urbanization, economic growth, community behavior, climate change, environmental sanitation conditions, and availability clean water (PHBS).

Fuzzy Geographically Weighted Clustering (FGWC) offers an alternative solution from regular clustering algorithms that better accommodate AGD with the ability to apply population effects and distances to geodemographic clustering analyses [3]. The previous study used FGWC integrates context-based clustering in its analysis, where CFGWC can reduce computing time and computing speed. It is expected that these results can accommodate spatial influence and also as alternative, which geographically aware by supporting the ability to apply population and distance effect to analyze geo-demographic clusters [4,5].

The purpose of this study is to integrate context-based clustering with FGWC-PSO to investigate clustering patterns of endemic areas in East Java based on the causes of dengue. Context-based clustering in this study was determined by FCM method. The result of this study will solve the problems in each cluster, which can be used by local governments to develop policies related to vector control strategies for the spread of dengue in East Java Province. The first government policy that can be implemented is by conducting a survey so that the policies to be carried out are more targeted.

MATERIAL AND METHOD

Study Area

The location of this research is East Java Province, which consists of 38 districts/cities. East Java Province geographically located between 7°12‘- 8°48‘ latitude and 111°0 ‘- 114^{0}4‘

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longitude. The total area of East Java Province is 47,799.75 km^2 [5]. The observation unit used is the district/city level in East Java Province, which consists of 38 districts/ cities.

Data Collection

This study uses secondary data on the factors that cause the spread of dengue hemorrhagic fever in 2017. The parameter used were percentage of dengue fever (X_1), percentage of bamboo walls (X_2), percentage of poor household (X_3), percentage of unhealthy houses (X_4), percentage of houses not PHBS (X_5), population density per Km^2 (X_6), percentage of rainwater shelter (X_7), number of flood events in a year (X_8), percentage of palm fiber roof (X_9), number of health facilities (X_{10}), number of cases of malnourished toddlers (X_{11}). Data was taken from the Central Bureau of Statistics (BPS) of East Java and East Java Province Health Office in 2018 [6,7]. Data on the population of districts/cities and distance of districts/cities also needed as supporting data for weighting.

Statistical Methods

Clustering dengue-endemic areas based on the factors that cause DHF spread using analysis from the CFGWC-PSO method. CFGWC-PSO method is a hybrid of the FGWC-PSO algorithm method and context-based clustering, which stands for CFGWC-PSO.

FGWC algorithm has several limitations in the initialization stage. First, the number of geodemographic clusters must be manually defined by the user. Second, the cluster center (centroid) is determined randomly so that the iteration process fails to reach the optimum global solution. To overcome this limitation, the PSO algorithm was used to select the center cluster or membership matrix in the FGWC initialization phase. FGWC objective function that will be minimized is as follows [8].

$$J_{FGWC}(U,V;X) = \sum_{i=1}^{c}\sum_{k=1}^{n} u_{ik}^{m}|v_i - x_k|^2 \rightarrow min \quad \text{.....1}$$

Where m is the weight exponent that determines the fuzziness clusters, u_{ik} is an element of the partition matrix, v_i is the center of the cluster, and x_k is the data point.

Objective function $J_{FGWC}(U,V;X)$ will be minimized by optimizing through parameters U and V. Lagrange multiplier λ_k with constraint $\sum_{i=1}^{c} u_{ik} = 1$ is used for find optimum value from u_{ik} and v_i.

Lagrange function for FGWC differentiates from each parameter and equal to zero for get the optimum value until get two formulations of objective functions as follows.

$$J_{FGWC}(V;X) = \sum_{i=1}^{c}\sum_{k=1}^{n}|v_i - x_k|^2 / \left(\sum_{j=1}^{c}\left(|v_i - x_k| / |v_j - x_k|\right)^{2/m-1}\right)^{m} \rightarrow min \quad \text{....2}$$

$$J_{FGWC}(U;X) = \sum_{i=1}^{c}\sum_{k=1}^{n} u_{ik}^{m}\left(\sum_{k=1}^{n} u_{ik}^{m}x_k / \sum_{k=1}^{n} u_{ik}^{m} - x_k\right)^2 \rightarrow min \quad \text{....3}$$

Both of these formulation processes are widely known as alternative optimization (AO), both used to optimize the FGWC model through several extreme conditions of the objective function FGWC (J_{FGWC}). The results of each function formulation are referred as FGWC-U and FGWC-V, while μ_{ik} in FGWC-U formula is a membership of the modified geographic cluster.

The method of context-based clustering is a method that gives a focus on data clustering based on special conditions or methods that centralize the original dataset by the specific conditions on its dimensions. Therefore, we found that only a portion of the original dataset with a relationship that matches the conditions specified. For dataset N with attributes $X = \{X_1, \dots, X_n\}$. Dataset will be classified into cluster C in dimensional space ($X_\varepsilon R^r$) with X_k is k data point and V_i is center of i-cluster. Context variables are defined as $Y \varepsilon X$

$$A: Y \rightarrow [0,1] \quad \text{....4}$$

$$y_k \rightarrow f_k = A(y_k) \quad \text{....5}$$

Where f_k represents the level of relationship between k-data point and i-cluster.

There are three methods in determining context variable such as random matrix with size $n \times 1$, using FCM, and calculate the average and standard deviation. In this paper, we used FCM method in determining the context variable [4].

Validity index is used to determine the right number of clusters. In this paper, we used Partition Coefficient (PC), Classification Entropy (CE), and Separation Index (S).

Equation of PC index, CE index, and S index can be described as follows

$$PC = 1/N \sum_{i=1}^{c}\sum_{j=1}^{N} \mu_{ij}^2 \quad \text{....6}$$

$$CE = -1/N \sum_{i=1}^{c}\sum_{j=1}^{N} \mu_{ij} log_a(\mu_{ij}) \quad \text{....7}$$

$$S = \sum_{i=1}^{c}\sum_{j=1}^{N} (\mu_{ij})^2 \|x_j - v_i\|^2 / N \quad \text{....8}$$

Where μ_{ij} is degree of j-data point membership in k-cluster, N is number of data points, v is cluster center, c is number of cluster, and x is data point.

RESULT AND DISCUSSION

Implementation of the FGWC-PSO algorithm using context-based clustering in many data packet analysis requires a number of clusters that can be determined by the researcher. Therefore, researchers tried by using the number of 2 clusters until 5 clusters. The calculation of the CFGWC-PSO algorithm evaluated by using three validity indexes, such as PC index, CE index, and S index, to find out the right number of clustering. The results of the validity index calculation showed in Table 1.

Table 1. Validity Index for Number of Clusters

C	CFGWC-PSO		
	PC Index	CE Index	S Index
2	**0.636053**	**0.545987**	**0.016137**
3	0.462956	0.914898	0.156111
4	0.472868	1.003822	0.154439
5	0.432561	1.141377	0.219087

Sources: Data Processing (2019)

Clustering performance measurement using the PC index, which is the greater the PC index value, indicates better clustering quality. CE index with an interpretation that is the smaller the CE index value is the better quality of clustering. S index has the same interpretation as the CE index. So, from Table 1, we can see that for the three validity index, the one that showed the best clustering is by using the number of clusters = 2. Therefore, for the next analysis, we used the number of clusters = 2. The number of districts/cities included in cluster 2 is more than in cluster 1 (Table 2). Cluster 1 has 11 districts/ cities and cluster 2 has 27 districts/cities.

Table 2. Results of Clustering

Cluster 1	Cluster 2	
Ponorogo	Pacitan	Lamongan
Lumajang	Trenggalek	Gresik
Jember	Tulungagung	Sampang
Banyuwangi	Blitar	Pamekasan
Bondowoso	Kediri	Sumenep
Situbondo	Malang	Kota Kediri
Probolinggo	Pasuruan	Kota Blitar
Mojokerto	Sidoarjo	Kota Malang
Jombang	Nganjuk	Kota Probolinggo
Bangkalan	Madiun	Kota Pasuruan
Surabaya	Magetan	Kota Mojokerto
	Ngawi	Kota Madiun
	Bojonegoro	Kota Batu
	Tuban	

Sources: Data Processing (2019)

Table 3. Average Clustering Variable Results

Cluster	X_1	X_2	X_3
1	1.2159908	**4.9536975**	2.273063
2	**4.1691160**	4.2481785	**3.030779**
JATIM	2.6925530	4.6009380	2.6519214
Cluster	**X_4**	**X_5**	**X_6**
1	26.0382	**47.1704**	**2051.83**
2	**32.4864**	42.8726	1828.79
JATIM	29.2623	45.0215	1940.31
Cluster	**X_7**	**X_8**	**X_9**
1	15.816268	3.118848117	0.0190863
2	**22.265694**	**3.658314014**	**0.0714926**
JATIM	19.040981	3.388581066	0.0452894
Cluster	**X_{10}**	**X_{11}**	
1	5.73963	**150.626**	
2	**11.1804**	91.7901	
JATIM	8.46004	121.208	

Sources: Data Processing (2019)

Table 3 showed that cluster 1 has four high-value variables that affect the factors causing the spread of DHF. Whereas cluster 2 has seven high-value variables that influence the causes of the DHF spread. Therefore, policies related to the efforts to control vectors, the handling of DHF focused on areas in cluster 2, especially on the factors that most influence the spread of DHF.

CONCLUSION

In this paper, we suggest that the policies related to efforts to control vectors in handling DHF focused on cluster 2 areas, especially on the factors that have the most influence on the spread of dengue. Problems in each cluster can be used by local governments to develop policies related to vector control strategies for the spread of dengue in East Java Province.

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The Antigenotoxic Activity of Brown Seaweed (*Sargassum* sp.) Extract Against Total Erythrocyte and Micronuclei of Tilapia (*Oreochromis niloticus)* Exposed by Methomyl-Base Pesticide

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Abstract

Pesticides are widely applied in the agriculture sector to protect crops and pest control. The poisonous substance of pesticides will affect all of the organisms, either target and non-target organisms. Fish can play the role of an indicator of genotoxic presence in aquatic environments. Polysaccharide extracts from sargassum have promising anti-genotoxic potential. This study aimed to analyze the anti-genotoxic activity of brown seaweed (*Sargassum polycystum*) methanol extract against erythrocyte and micronuclei of tilapia exposed by methomyl-base pesticide. Brown seaweed (*Sargassum* sp.) purchased from farmers in Sumenep Regency, Madura, East Java, then macerated using methanol 1: 3 (w / v) for 3x24 hours at room temperature. The phytochemical screening was including flavonoids, alkaloids, triterpenes, saponins, and tannins. Tilapia fish (TL ± 9-12cm) purchased from the Technical Application Unit of Freshwater Fish (UPT Perikanan Air Tawar), Sumberpasir, Malang, East Java. The result of this study showed that exposure of methomyl-based pesticides in the concentration of 4.015 ppm indicates the formation of micronuclei of 318.33 ‰. The increased concentration of extract treatment is directly proportional to the decrease of micronuclei. It means that sargassum extract can reduce the genotoxic effect on exposed tilapia by methomyl-based pesticides. The best concentration of *Sargassum* sp. extract that can reduce genotoxic was D (200 ppm).

Keywords: Antigenotoxic, Extract, Methomyl, Pesticides, *Sargassum* sp., Tilapia.

INTRODUCTION

Pesticides are widely applied in the agriculture sector to protect crops and pest control [1]. Methomyl ($C_5H_{10}N_2O_2S$), S-methyl-1-N- [(methyl carbamoyl)-oxy]-thioacetimidate, is a carbamate pesticide which widely used in many agricultural countries because of its high-efficiency biological activity to control pest and protect the crops [2,3]. Some pesticides aimed to attack a specific group of the target, but its poisonous substance will affect all of an organism, either organism target and non-target [4]. On another side, the specific concentration of methomyl pesticides can cause acute poisoning, even death of fish [5]. A study report that methomyl pesticide caused a genotoxic effect against fish [6].

Aquatic environments are the final sink for many chemicals, including pesticides, and water can serve as the vehicle for exposure to many toxic agents [7]. Fish are the principal of aquatic animals. They can be an excellent sample for monitoring pesticide toxicity, such as methomyl in aquatic systems. It is because they are susceptible to pollutants, they can metabolize xenobiotics, and they relatively exhibit a very high bioaccumulation rate of dissolved chemicals [8-11].

The genotoxic effect is a change in the function of gene expression that occurs due to a bond between DNA and carcinogenic substances. It leads to the appearance of physiological changes in the body, such as chronic tissue damage, changes in the body's immune system, hormonal changes, or binding to proteins that are repressive to specific genes [12]. To overcome the genotoxic effects due to exposure to a toxin, such as pesticides, is through the administration of extracts that function as antigenotoxic effects. Several studies report that polysaccharide extracts from sargassum have promising anti-genotoxic potential and anti-mutagenic activity [13,14]. This study aimed to analyze the anti-genotoxic activity of brown seaweed (*Sargassum polycystum*) methanol extract against erythrocyte and micronuclei of tilapia, exposed by methomyl-base pesticide.

MATERIAL AND METHOD

Preparation of Seaweed Extract

Brown seaweed (*Sargassum* sp.) purchased from farmers in Sumenep Regency, Madura, East Java. The seaweed cleaned by using freshwater

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then shade-drying for four days. Dry seaweed then ground until seaweed powder obtained.

Total of 500 g seaweed powder put into a jar, then macerated using methanol 1: 3 (w / v) for 3x24 hours at room temperature, in three times replication. The maceration solution is then filtered using filter paper (Whatman no. 41), then filtrate and residue obtained. The filtrate then evaporated using a rotary evaporator vacuum at 40°C until a concentrated extract obtained then calculated the percentage of yield.

Phytochemical Screening

Phytochemical screening, including flavonoids, alkaloids, triterpenes, saponins, and tannins, followed the published methods [15].

Determination of Flavonoids: seaweed powder (1 g) was boiled with 10 mL of distilled water for 5 minutes and filtered while it was hot. A few drops of 20% sodium hydroxide solution added to 1 mL of the cooled filtrate. A change to a yellow color, in addition to acid, changed into a colorless solution, that depicted the presence of flavonoids [15].

Determination of alkaloids: the seaweed powder (200 mg) boiled with 10 mL water and 10 mL of hydrochloric acid using a water bath. Finally, it was filtered, and its pH adjusted to about 6-7 with ammonia. The filtrate (1 mL), add with a few drops of Mayer's reagent (potassium mercuric iodide solution). Besides, a 1 mL portion was treated similarly with Wagner's reagent (solution of iodine in potassium iodide). Turbidity or colored precipitation with either of these reagents was taken as evidence for the presence of alkaloids [15].

Determination of triterpenes: A total of 3 g of seaweed powder placed into a test tube, then add 10 mL of 50% alcohol, heated for 3 minutes on a water bath. After that, cool at room temperature then filtered. The filtrate evaporated in an evaporating dish. Petroleum ether (5 mL) then added to the dish, stirred for 5 minutes. We added chloroform 10 mL and stirred for about 5 minutes, then transferred into a test tube, and 0.5 mg of anhydrous sodium sulfate was added and shaken gently then filter. The filtrate was then divided into two test tubes and used for the following tests.

Liebermann-Burchard's reaction: To test tube I, an equal volume of acetic anhydride added and gently mixed. Then 1 mL of concentrated sulfuric acid was added down the side of the tube. The appearance of a brownish-red ring at the contact zone of the two liquids and a greenish color in the separation layer indicates the presence of sterols and triterpenes.

Salwoski's test: To test tube II, 2 to 3 drops of concentrated sulphuric acid was added to form a lower layer. The reddish-brown color at the interphase indicates the presence of a steroidal ring [15].

Test for saponins: seaweed powder (1 mL) was diluted with distilled water to 20 mL and shaken for 15 minutes in a graduated cylinder. The development of stable foam suggests the presence of saponins [15].

Test for tannins: Sample (1 g) was boiled with 20 ml distilled water for 5 min in a water bath and filtered while it was hot. Then 1 ml of cool filtrate was diluted to 5 ml with distilled water, and a few drops (2,3) of 10% ferric chloride were added and observed for the formation of precipitates and any color change. A bluish-black or brownish-green precipitate indicated the presence of tannins [15].

Animal Preparation

Tilapia fish (TL ± 9-12cm) purchased from the Technical Application Unit of Freshwater Fish (UPT Perikanan Air Tawar), Sumberpasir, Malang, East Java. Fish then acclimatized in the aquarium based on published methods [6].

Pesticide Preparation

The methomyl-based pesticide purchased from the agriculture market in Batu, East Java, Indonesia, as Lannate 25 WP. The concentration of methomyl-base pesticides in this study was 4.015 ppm base on a published report [6].

Treatment

Research phase 1: Prepare the aquarium (60x30x30cm) labeled for control (without treatment) and test (three replication each) then refill with fresh water. Acclimated tilapia fish then transferred into the prepared aquarium, ten fishes each. Dissolve methomyl-based pesticide that has been measured into each aquarium, allow up to 2 x 24 hours then sampling the fish blood in each aquarium.

Research phase 2: Prepare the aquarium (60x30x30cm) labeled for test and control (three replication each) then refill with fresh water. Transfer the remaining fish gently from research phase 1 into aquarium research phase 2 then dissolve extract of sargassum (Negative Control, 25 ppm, 50 ppm, 100 ppm, 200 ppm, and positive control) modified from the published concentration [16], allow up to 2 x 24 hours then sampling the fish blood in each aquarium.

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Data Collection

Total Erythrocyte Count

The tilapia anesthetized using a benzocaine solution (1g.10L^{-1} water). After immobilization, around 2.0 mL of blood withdrew using puncturing at the caudal peduncle, using syringes and sterilized needles. One drop of the blood sample smeared on object-glass, then air-dry and stained using Rosenfeld's procedure [17] for 10 minutes, so a blood sample preparation obtained. Erythrocyte counts performed according to a published method with an optical microscope [18]. The results obtained were subjected to analysis of variance. The F values that indicated significant differences ($P < 0.01$) were subjected to the Duncan test to compare the means.

Micronuclei Assay

After 96 hours exposed by methomyl-based pesticide, erythrocyte blood from each fish group sampled and smeared on clean microscope slides. After fixation in absolute methanol for about 20 min, the slides air-dried and stained with 10% of Giemsa for about 25 minutes. Six slides of 1,000 erythrocytes that sampled from each Tilapia (*O. niloticus*) scored [19], observed, and coded by using a microscope (Olympus CX21) with 400X magnification. It determined the frequency of micronucleus cell and other different patterns of morphologically altered erythrocyte and then counted as cell per 1000 (‰) [20]. The micronucleus frequency then counted base on Betancur formulation [21].

$$\text{Micronuclei frequency} = \frac{\text{Micronuclei x 1000}}{\text{Total Cell Counted}}$$

RESULT AND DISCUSSION

Extract yield

Quantification result of *Sargassum* extract yield at repetition 1, 2, and 3 was 9%, 7.22%, 8.09%, respectively (Table 1). The yield is an important parameter to determine the economic value and effectiveness of an ingredient or product. A yield is a percentage of the raw material that can be utilized. The higher the yield value of a material, the higher its economic value as well as its utilization.

Table 1. Yield extract of *Sargassum* sp.

Repetitions	Yield weights (Gram)	Percentage (%)
1	8.,83	9.00%
2	7.22	7.22 %
3	8.09	8.09 %

Sargassum sp. extract different from the yield of *S. filipendula* extract with ethanol solvent in the other that is 1.69 ± 0.159% [22]. The use of methanol solvents was more effective in the extraction of red algae *Kappaphycus alvarezii* and *Eucheuma denticullatum* than ethanol, which had a lower polarity level [23]. The difference in extract yield depends on the natural conditions of the sample, the extraction method, the particle size of the sample, the conditions and the extraction time, and the comparison of the sample with the solvent [24].

Phytochemical screening

Phytochemical data shows the chemical compositions in constituents of the sargassum extracts. The results of the phytochemical evaluation showed in Table 2. Phytochemical testing is necessary because it aims to determine the right type of secondary metabolic components in various plants, such as *Sargassum* sp. [25].

Table 2. Chemical compositions in *Sargassum* sp. extract

Constituents	Result
Flavonoids	+
Alkaloids	-
Triterpenes	+
Saponins	+
Tannins	+

The phytochemical screening on *Sargassum* sp. showed the existence of compounds of Flavonoids, Triterpenes, Saponins, and Tannins. Alkaloids were absent in this seaweed. These plants contain essential phytochemical constituents and have various potential biological activities [26]. Phytochemical results of the current work revealed that these edible seaweeds could be potential candidates in the field of drug development.

Hematological Analysis Result

Total Erythrocyte Count

The erythrocyte count of healthy fish (positive control) showed a mean value of 6.65 x 10^6.mm^{-3}, and the exposed fish by pesticide without extract treatment (negative control) showed a mean value of 1.67 x 10^6.mm^{-3}. The sargassum extract treatment to exposed fishes by methomyl-based pesticide with concentration A (25 ppm), B (50 ppm), C (100 ppm), and D (200 ppm) showed mean value of total erythrocyte count as 1.67, 1.69, 3.00, and 5.30 x 10^6.mm^{-3}, respectively. The values mentioned in Figure 1 showed a significant increase when compared to the negative control.

According to figure 1, treatments fed with the algae extract exhibited significant increases in the WBC count. The increased concentration of extract treatment is directly proportional to the increase of erythrocyte cells. The erythrocyte

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count is an essential indicator of the internal and external environment of animals, where exposure to chemical pollutants can induce either increases or decreases in hematological levels [27].

The use of immunostimulants such as algae extract will be improving the immune system in fish. The fish immune system reflects the role of dietary supplements in boosting immunity because of specific characteristics, including a more efficient non-specific vs. specific immunity compared to those of warm-blooded animals [28]. Due to increased resistance of fish against environmental stress and pathogens [29], the use of immunostimulants is a widely examined approach to increase production [30,31] in intensive and ultra-intensive rearing conditions.

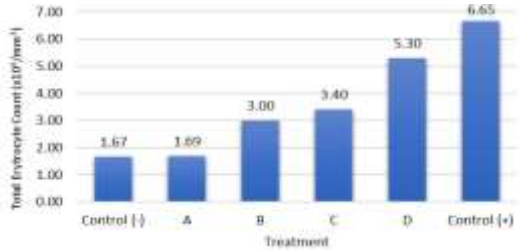

Figure 1. The Total Erythrocyte Count of Tilapia

Micronuclei assay

The Micronuclei assay in fish erythrocyte is widely used for genotoxicity assessment of marine and freshwater organisms. The frequencies of micronuclei formation at negative control (exposed fish without extract treatment) were significantly ($p<0.05$) higher than the positive control (healthy fish) at treated groups. The genotoxicity of pesticides on fish in the present study was found in peripheral blood erythrocytes [6]. The micronuclei test is a sensitive assay to evaluate genotoxic compounds present in fish [32]. Peripheral blood erythrocytes (Fig. 2) of fish were for most of the micronuclei surveys, that carried out in peripheral blood erythrocytes of fish [33,34].

The micronuclei assay of tilapia fish during the research shown in Figure 3. The exposure of methomyl-based pesticide (control negative) indicates the formation of micronuclei 318.33 ‰. The increased concentration of extract treatment is directly proportional to the decrease of micronuclei. It means that sargassum extract can reduce the genotoxic effect on exposed tilapia by methomyl-based pesticides. Marine algae extract proved to be a powerful anti genotoxicant, which is primarily due to the combined role of phenolics and flavonoids [14].

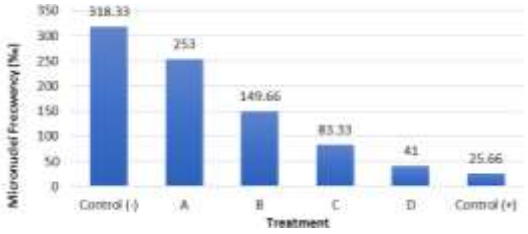

Figure 2. Micronuclei assay test in erythrocyte of tilapia

Dietary flavonoids have protective effects against DNA damage induced by different genotoxic agents. Flavonoids exert their genoprotections by reducing oxidative stress and modulation of enzymes responsible for bioactive of genotoxic agents and detoxification of their reactive metabolites [35]. Results showed that the best concentration of *Sargassum* sp. extract that can reduce genotoxic was D (200 ppm).

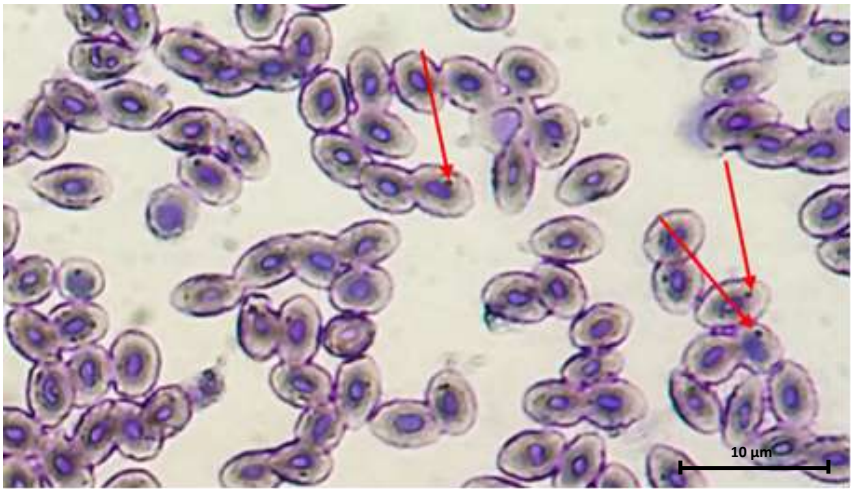

Figure 3. The micronuclei in erythrocyte cell (red arrows)

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CONCLUSION

The exposure of methomyl-based pesticides in the concentration of 4.015 ppm indicates the formation of micronuclei of 318.33 ‰. The increased concentration of extract treatment is directly proportional to the decrease of micronuclei. It means that sargassum extract can reduce the genotoxic effect on exposed tilapia by methomyl-based pesticides. The best concentration of *Sargassum* sp. extract that can reduce genotoxic was D (200 ppm).

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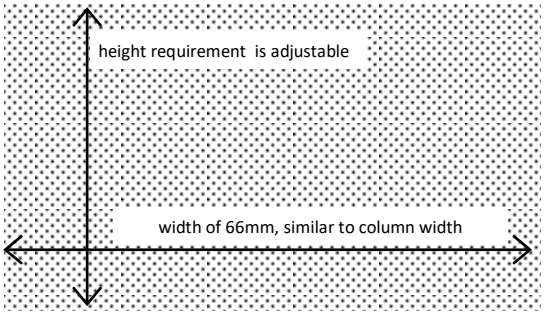

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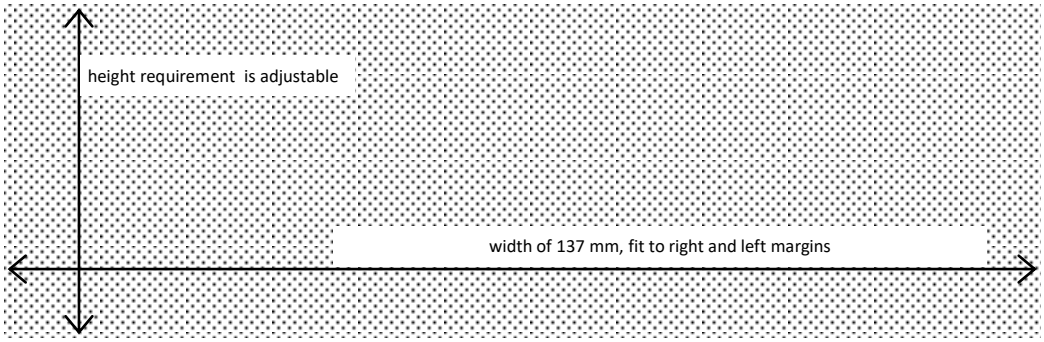

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References

1. Primary references include journal, patent, dissertation, thesis, paper in proceeding and text book.
2. Avoid self citation.
3. Author should avoid reference in reference, popular book, and internet reference except journal and private ana state institution.
4. Author was not allowed to use abstract as references.
5. References should been published (book, research journal or proceeding). Unpublished references or not displayed data can not be used as references.
6. References typed in numbering list (format number 1,2,3,…), ordered sequentially as they appear in the text (system of Vancouver or author-number style).
7. Citation in the manuscript typed only the references number (not the author and year), example: Obesity is an accumulation of fat in large quantities which would cause excessive body weight (overweight) [1]. Obesity is a risk factor of diabetic, hypertension dan atherosclerosis [2].

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)

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Cover Images:

3D Structure of EGCG (*Epigallocatechin-3-Gallate*)
Green Tea Component

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