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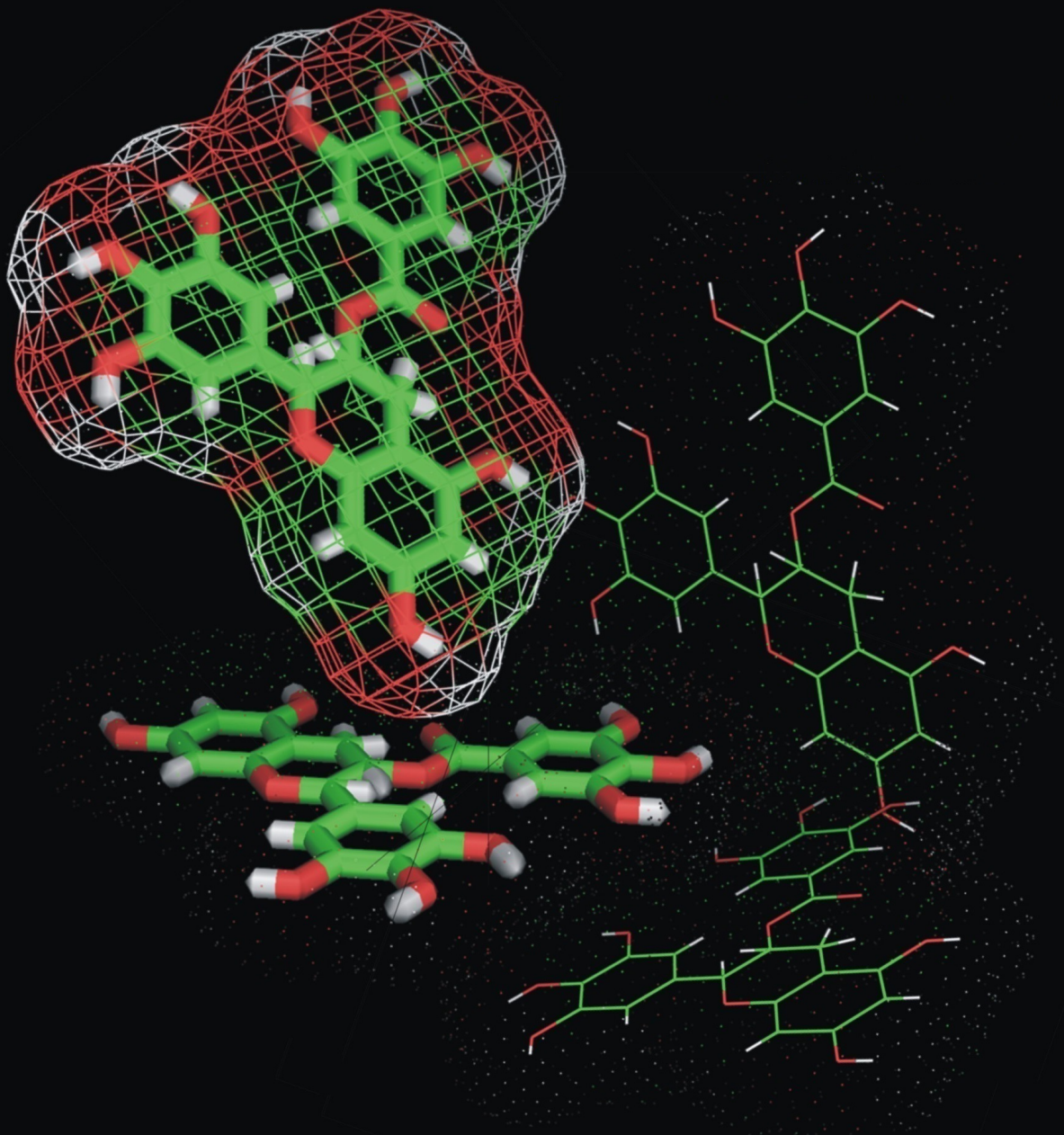

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Phytochemical Screening and Antimicrobial Activity of Roselle (*Hibiscus sabdariffa* L.) Flower Extract Against *Aeromonas hydrophila*

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Abstract

Medicinal plants as an antimicrobial agent may provide an alternative way to replace the use of antibiotics to control disease agents in aquaculture. Roselle flowers (*Hibiscus sabdariffa* L.) has been used in many sectors as a source of functional food, natural coloring agents as well as antimicrobial agents. The objectives of this study were to evaluate the phytochemical compound in methanolic extract of roselle flower and their antimicrobial activity against *Aeromonas hydrophila*. The phytochemical composition of roselle flower was evaluated using phytochemical screening and FTIR. While the antimicrobial activity was performed by using the disc diffusion agar and co-culture with *A. hydrophila*. The results of phytochemical screening confirmed the presence of alkaloids, flavonoids, saponins, steroids, triterpenoids, and tannins. The results of FTIR revealed that Roselle flower extract had the main phenolic compounds. The result of disc diffusion and co-culture method indicated that the roselle flower extract had antibacterial activity against *A. hydrophila*. This antibacterial activity depended on the concentration applied.

Keywords: antibiotics, Co-culture, Disk diffusion, flavonoid, *Hibiscus* sp.

INTRODUCTION

Aeromonas hydrophila is a common bacteria in freshwater habitats throughout the world [1]. It cause diseases both in human and animals including fish and shrimp [2]. Bacterial infections with *Aeromonas hydrophila* as a disease agent have resulted in heavy losses up to 80% and economic loss to fish farmers [3,4].

Usually, many farmers have used antibiotics to control this disease. However, antibiotics have become the major factor for the emergence and dissemination of multi-drug-resistant strains of several groups of microorganisms [5]. An effort to overcome the negative effects of antibiotic application is the use of medicinal plants. Plants are rich in a wide variety of secondary metabolites such as *tannins, alkaloids*, and *flavonoids*, which have been found in vitro to have antimicrobial properties [6]. There have been several reports on the antimicrobial activity of different herbal extracts [7–9].

Hibiscus sabdariffa L., well-known as roselle, is a common flower plant grown worldwide. More than 300 species of hibiscus can be found around the world [10]. Roselle have been used in many sectors as a source of functional food, natural coloring and antimicrobial agents [11–13]. Several studies found that roselle flower have significant antimicrobial activities against several strain of bacteria such as *Micrococcus luteus, Serratia mascences, Staphylococcus aureus, Staphylococcus epidermidis, Salmonella enterica*, *Pseudomonas aeruginosa, Klebsiella pneumonia, Proteus vulgaris, Escherichia coli,* and *Bacillus cereus* [14,15]. Yet, no studies found to reveal the antimicrobial activities against *A. hydrophila* as a bacterial disease agent in aquaculture. Therefore, this study was intended to evaluate the phytochemical screening and antimicrobial activity of Roselle flower extract on *A. hydrophila* indigenous aquatic.

MATERIAL AND METHODS

Preparation of Roselle flower Extract

Roselle flower (*H. sabdariffa* L.) was originated from house of medicinal plant, Batu, East Java. The flowers were air-dried and grounded into fine powder using an electric blender. Extraction process was performed based on the methods of [16] with some modification. A hundred (100) g of air-dried powder was mixed with 500 ml of methanol solvent and then was kept for 24h. Later, it filtered through Whatman filter paper (no. 42) and centrifuged at 5.000 g for 10 min. The extract was evaporated using a rotary evaporator at 40°C.

Phytochemical Composition and FTIR of Roselle Flower Extract

Phytochemical screening was carried out to confirm the presence of alkaloids, flavonoids, saponins, triterpenoids, steroids, and tannins using the standard method [17]. FTIR analysis was also conducted to elucidate the

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phytochemical composition of roselle flower extract based on standard methods [18]. Roselle flower extract (1 mg) was homogenously mixed with 100 mg KBr and pressed in to pellet. FTIR spectra were recorded in the range 4000-400 cm-1 in FTIR spectroscopy (OPUS 4.2, Karlsruhe, Germany).

Bacterial preparation

Isolate of *Aeromonas hydrophila* was originated from Jepara Brackishwater Aquaculture Center. This bacterium was kept in *Trypticase Soy Agar* (TSA) media at 4°C and sub-cultured *Trypticase Soy Broth* (TSB) in overnight before use.

Antimicrobial assay

Concentration of Roselle flower extract used in this study were 0, 1, 10, 100, 500 and 1,000 $mg.ml^{-1}$ which diluted in 10% DMSO. Antimicrobial assay was carried out using modified disc diffusion method and co-culture method. The modified disc diffusion method was performed based on [19]. The overnight bacterial suspension was adjusted to the concentration of 10^7 $CFU.mL^{-1}$ and seeded (0.1 mL) on Muller Hinton Agar (MHA) plate. Each treatment (5 µL) of Roselle flower extract was applied in to MHA plate. A positive control antibiotic (Chloramphenicol 5 $mg.mL^{-1}$) and a negative control (without extract) was used in this assay. MHA plates were incubated at 37°C for 24 hours. Antimicrobial activities was evaluated by measuring the inhibition zone. The co-culture method was performed based on [20]. In 100 mL TSB, *A. hydropila* (1 mL) was inoculated with an initial bacterial count of 10^7 $CFU.mL^{-1}$. The flower extract was added in to the flask at the varied concentration above. Those co-culture flasks were incubated at 37°C on shaker (120 rpm) for 24 hours. The *A. hydrophila* was enumerated by using the standard plate method. All treatments were performed in triplicates.

RESULT AND DISCUSSION

Phytochemical Composition

The phytochemical analysis was performed to confirm the presence of alkaloids, flavonoids, saponins, steroids, triterpenoids and tannins in the extract of roselle flower. The results of phytochemical analysis were given in the Table 1. The results showed that all the compound tested were found in roselle flower extracted with methanol solvents. Results of this phytochemical analysis was also supported by the earlier studies [21]. They reported that the flower of roselle contained group of alkaloids, flavonoids, saponins, steroids, triterpenoids and tannins. Other plants that have antimicrobial activities were also had these compounds [22–24].

Table 1. The Phytochemical Analysis of Roselle Flower Extract

Metabolites	Results
Alkaloids (Dragendroff)	+
Alkaloids (Mayer)	++
Flavonoids	+++
Saponins	+
Steroids	+
Triterpenoids	+
Tannins	+

Notes: +++ = strong, ++ = medium, + = weak intensity [25]

FTIR Analysis Results

The spectrum of FTIR was designed to identify the functional group of bioactive compound. This assignment was confirmed based on the peaks value in the IR radioation region. The FT-IR spectrum of roselle flower extract is presented in Fig. 1, while the spectra is interpreted in Table 2. The results indicated that there were various functional groups present in the roselle flower extract.

Table 2. Assignment of FT-IR Absorption Bands in the Roselle Flower Extract

Absorption frequency (cm^{-1})	Bond	Tentative assignment
3,419.71	O-H	Alcohol, phenol
2 928.91	C-H	Alkanes
1 743.03	C = O	Aldehydes
1,630.18	C = C	Alkenes
1,384.12	C-H aliphatic	Alkanes
1,228.37	C-O	Carboxylic acid
1,065.59	C-O	Carboxylic acid
523.34	C-H	Aromatics

The result of FTIR analysis confirmed the presence of phenol, alkanes, aldehydes, carboxylic acid, and aromatics. Allegation that the results of the isolate are phenolic compounds where the benzene group binds to one OH group with a wide and sharp inclination with absorption at the wave number area 3419.71 cm^{-1} and 1384.12 cm^{-1}. And reinforced functional groups OH, C = C, C = O, C-H aromatic [26–28]. Based on the results of phytochemical screening analysis and FTIR analysis, it can be linked that flavonoids and positive tannins contained in roselle flower extract are derivatives of phenol [29,30]. According to previous study [31], phenol derivatives contained in plants have antimicrobial activity.

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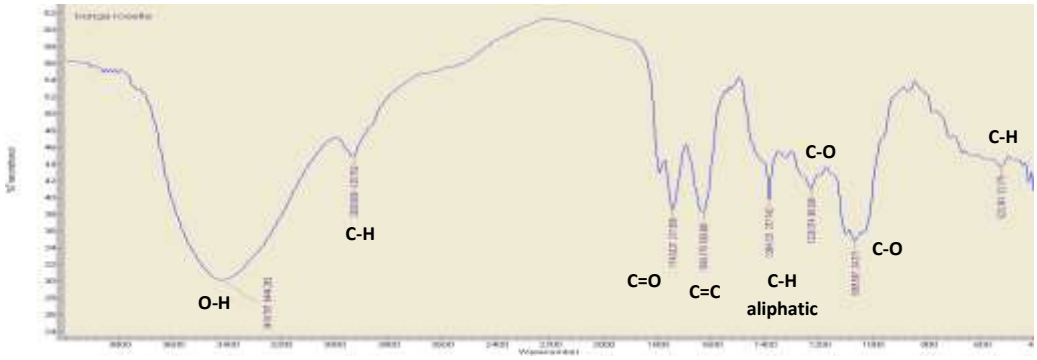

Figure 1. FTIR Test Results of Rosella Flower Extract (H. sabdariffa L.).

Antimicrobial Assay

The results of antimicrobial assay of *H. sabdariffa* L. provide valuable information and highlight the potential value of this plant in aquaculture drug development. Classification was made based on the width of the clear zone formed. The results of the antimicrobial assay of the methanolic extract of *H. sabdariffa* L. against *A. hydrophila* can be seen in Table 3.

Table 3. Antimicrobial Testing of Roselle Flower Extract against *A. hydrophila* by Using Disc Diffusion

Concentration of *Roselle flower extract* (mg.L^{-1})	Clear Zone Diameter (mm)	Inhibitory Responses
Control (-)	0.00±0.0^{a}	Weak
1	0.00±0.0^{a}	Weak
10	2.19±0.0^{b}	Weak
100	7.12 ±0.8^{c}	Medium
500	15.02±0.5^{d}	Strong
1000	18.37±0.3^{e}	Strong
Control (+)	21.47±0.7^{f}	Strong

Description: ≤5mm =weak, 5-10 mm= medium,10-20 mm= strong [32]

Table 4. Co-Culture Test Result

No	Concentration of *Roselle flower extract* (mg.L^{-1})	Bacterial count (CFU. mL^{-1})
1	Control (-)	2.70 E18
2	1	2.66 E18
3	10	1.85 E14
4	100	1.08 E11
5	500	8.6. E09
6	1000	5.3. E08
7	Control (+)	0

The result of disc diffusion and co-culture method indicated that the roselle flower extract had an antimicrobial activity against *A. hydrophila* (Table 4). Some studies also revealed the antibacterial activities of Roselle extract against some bacteria such as *Streptococcus mutans* [33], *Campylobacter jejuni, C. coli, C. fetus* [34]. The antibacterial activities detected in this study were concentration-dependent. The higher the concentration of Roselle flower extract, the higher the antibacterial detected against *A. hydrophila*. It can be seen from Table 3 that the concentration of roselle flower extract which categorized withs strong ability to inhibit the growth of *A. hydrophila* was started from 500 mg.L^{-1}. In line with that finding, the results of co-culture assay showed that the significant reduction of *A. hydrophila* count was also from the same concentration. The antimicrobial activity was influenced by several factors such as the concentration of extract, the content of antibacterial compounds, the diffusion power of extracts and the type of bacteria [35].

The ability of Roselle flower extract to inhibit the growth of *A. hydrophila* depend on their bioactive compound. The main compound of Roselle are organic acids, anthocyanins, polysaccarides and flavonoids [36]. Based on the results of the phytochemical screening test and FTIR analysis in this study, it was found that Roselle flower extract had the main phenolic compounds such as flavonoids, tannins. These phenolic compounds have the ability to form certain complex structures on bacterial cell walls. Furthermore, with the number of hydroxyl groups present in the phenolic ring there will be an increase in hydroxylation, and with increased hydroxylation, antimicrobial activity will increase [37].

Other compounds which were also found in Roselle methanol extract are alkaloids, saponins, steroids, and triterpenoids. Those compounds have also antimicrobial activities by damaging the structure of cell walls and changing the permeability of cell cytoplasmic membranes [38], [39]. Changes and damage to the cytoplasmic membrane cause leakage of intracellular material and cell metabolic disorders [40].

CONCLUSION

The phytochemical analysis of methanol extract roselle flowers confirmed the presence of

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alkaloids, saponins, steroids, triterpenoids, tannins and strong intensity of flavonoids. The results of FTIR revealed that Roselle flower extract had the main phenolic compounds. The antimicrobial assay found that the roselle flower extract had antibacterial activities against *A. hydrophila*. The antibacterial activities depended on the concentration of Roselle flower extract.

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Phytochemicals and The Ability of *Plantago major* Linn. Extract to Inhibit The growth of *Aeromonas hydrophila*

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Abstract

Aeromonas hydrophila is a type of gram-negative bacteria that can harm humans and major animals that have poikilotherm properties such as fish and shrimp. These bacteria can cause haemorrhages in fish until death and causing fish farming production to failure. The use of medicinal plants has been trusted by people all over the world to overcome various disease problems, one of which is a disease caused by bacteria. *Plantago major* L. is known that able to inhibit the growth of both gram-positive and gram-negative bacteria. The purposes of this study were to identify the extracted compounds contained in *Plantago major* L., using FTIR and UV-VIS spectrophotometer and to evaluate antibacterial activity against A. *hydrophila*. The results of this study showed that crude extracts of *Plantago major* L. contained polar compounds such as phenols, flavonoids, saponins, and tannins. The presence of phenols, tanin and flavonoids was confirmed by the results of FTIR and UV-VIS. Furthermore, the crude extract of *Plantago major* L. significantly inhibited the growth of A. *hydrophila* ($P < 0.05$).

Keywords: Antibacterial, Co-culture, Extraction, FTIR, UV-VIS.

INTRODUCTION*

Aeromonas hydrophila is a type of bacteria that was widespread in both fresh and brackish water environments. These bacteria can infect various animals especially animals that are poikilotherm, such as fish and shrimp. If these animals are infected by the bacteria, the quality of their meats will be reduced [1,2,3]. *A. hydrophila* is known as an agent of *Motile Aeromonas Disease* or MAS, present as secondary pathogens in fish that have been exposed to environmental stress. The symptoms of MAS were observed as haemorrhages and pathological damages in internal organs. Infected fish experienced mortality up to 80-100% in a short time[4,5,6].

The use of medicinal plants has been known and trusted by 80% of the World community. *Plantago major* L. is a well-distributed plant over the World and known as a 'weed' that live freely in nature [7]. In Indonesia, 28% of *Plantago major* L. is found growing wild on the roadside and used as a wound medicine [8]. It has an ability to cure white discharge problem [9], inflammation and coughing [10]. This weed was known to have several activities such as antibacterial, antifungal and antiviral agents as well as antioxidant and analgesic [11]. The previous study affirmed the benefits of *Plantago* major L. in the field of health because of several compounds such as polyphenols, alkaloids, tannins, and steroids [12].

As an antibacterial agent, *Plantago major* L. extract was able to inhibit gram-positive and gram-negative bacteria such as *Lactobacillus* sp., *S. aureus, E. coli, P. aeruginosa*, *K. pneumonia*, *Proteus* sp., S. *enteriditis* [13,14]. However, there is no report on the ability of *Plantago major* L. to inhibit the growth of A. *hydrophila*. Therefore the aim of this study was to determine the phytochemical content of *Plantago major* L. and its ability to inhibit the growth of A. *hydrophila*.

MATERIAL AND METHODS

Phytochemical Extraction and Analysis

The Phytochemical analysis was carried out by referring to the method of [15], to observe the content of phenols, flavonoids, alkaloids, tannins, saponin, terpenoids (steroids and triterpenoids). Plants of Plantago major L. aged 3 – 4 months were obtained from Materia Medica in Batu, dried for 5 days and made into powder using blender according to the method of [16], with modification. *Plantago majo*r L. powder (200 g) was macerated with 1L of ethanol PA (1:5) for 5 days. The results of maceration were filtered using Whatman paper No. 42 and evaporated using a rotary vacuum evaporator at 50°C, 65 rpm to obtain a thick green extract.

The extract was analysed using FTIR and UV-Vis spectrophotometry. FTIR is used to determine the functional group or type of the active compound based on the peak value of the

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wavelength. FTIR testing was conducted referring Jain et al. [17]. The obtained extract was mixed with KBr in a mortar and pressed with a pressure of 6 bars for 2 minutes. Samples were scanned on a spectrometer IR (FT 1000 Varian) 4000 - 400 cm^{-1}.

UV-VIS analysis was carried out by using UV-Visible spectrophotometry (Cary50 Conc Varian) [17]. Extract *Plantago major* L. was diluted using ethanol (1:10), then examined under UV wavelength 300 - 800 nm.

Antibacterial Test

This research was carried out at the Fish Health and Disease Division Aquaculture Library, Faculty of Fisheries and Marine Science, Brawijaya University from November – December 2018. A bacterial isolate of A. *hydrophila* was obtained from BBPBAT Jepara, Central Java. The bacteria was rejuvenated with TSA medium and re-cultured in TSB medium with a bacterial density of 2.35 x 10^7CFU.mL^{-1}. TSA medium that used for antibacterial activity was 20 ml with spread method. Antibacterial activity tests were carried out using a disc diffusion method [18].

Blank disk with a size of 6 mm was dipped into the crude extract which has been diluted with 10% DMSO. The doses used were 100, 200, 300, 400, 500 mg.L^{-1}, with DMSO 10% as negative controls and Chloramphenicol as positive controls. All the treatments then incubated at the incubator 37°C for 24 hours. The diameter of the inhibition zone is measured using a caliper. Furthermore, co-culture tests were used to confirm the results of antibacterial activity by referring to the method [19] where bacterial culture and various concenterations of plant extracts were mixed in tubes containing sterile TSB medium, then incubated for 24 hours. The cultures were then transferred in TSA plates and bacterial density was enumerated after 24 hours.

RESULT AND DISCUSSION

FTIR Analysis

The FTIR results of *Plantago major* L. and the absorption peaks are presented in Figure 1 and Table 1. From Figure 1, there are six types of absorption some of which are absorption at waves 3854.124 cm^{-1} indicating that there is an O–H group, the changes in wave 3740.940 cm^{-1} detected the presence of O–H groups, absorption at wave 3446.329 cm^{-1} shows the presence of O–H groups with stretching vibrations, at wave 1640.934 cm^{-1} detected a C=C group with stretch vibration, the strong absorption band at wave 1384.402 cm-1 was found to be a C–H group and the absorption of waves of 1050.935 cm^{-1} was found to have a C–O bond with strong intensity. Stretch bands of phenyl groups C=C, –OH, and –CH are characteristics of IR which indicate the presence of phenol and flavonoid and tannin compounds [20-23]. This absorption of the IR spectrum is similar to a previous study, which states that *Plantago major* L. contains a carboxyl, hydroxyl, and methyl group [24].

Tabel 1. FTIR Peak Result of *Plantago major* L.

No.	Peak Values (cm^{-1})	Functional Group
1.	3854.124	O – H carboxylic acid
2.	3740.940	O – H alcohol
3.	3446.329	O – H alcohol, fenol
4.	1640.934	C = C alkena
5.	1384.402	C – H alkana
6.	1050.935	C – O alcohol

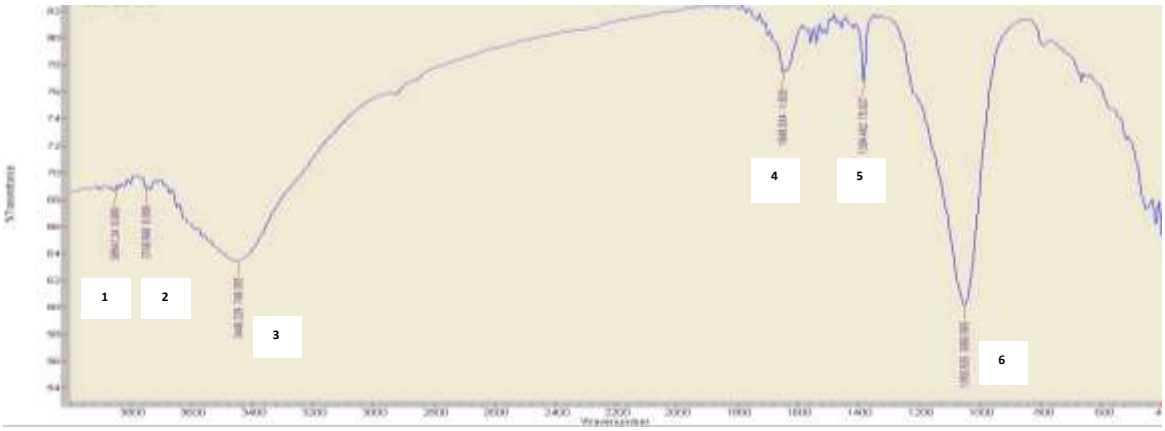

Figure 1. FTIR Test Results of *Plantago major* L.

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UV-VIS Analysis

UV-Vis results of *Plantago major* L. ethanol extract presented in (Fig. 2). The UV spectrum in the maximum wavelength at 227 nm, 411 nm, and 505 nm strongly suspected contained phenol compounds that are flavonoids. The main flavonoids presents are flavones, flavonol, and auron. This was confirmed that flavones were the main type of flavonoids in *Plantago major* L. [25]. There are several flavones in *Plantago major* L. but only one flavonol was detected in UV-spectophotometry [26].

Phytochemical Extraction and Analysis

Result of the phytochemical analysis showed that crude extract of *Plantago major* L. contained phenols, flavonoids, saponins, and tannins compounds. On the other hand, there were no alkaloid and terpenoid compounds (Table 2). However, another study stated that *Plantago major* L. extract contained a composition of alkaloids and terpenoids [25]. Phenols, flavonoids, saponins, and tannins are polar compounds which have various functions, one of which is an antibacterial activity [27].

Tabel 2. Phytochemical test result of *Plantago major* L.

No.	Compound	Result
1.	Fenol	+
2.	Flavonoid	+
3.	Alkaloid	
	Mayer	-
	Dragendrof	-
	Bouchardat	-
4.	Saponin	+
5.	Tanin	+
6.	Terpenoid	
	Steroid	-
	Triterpenoid	-

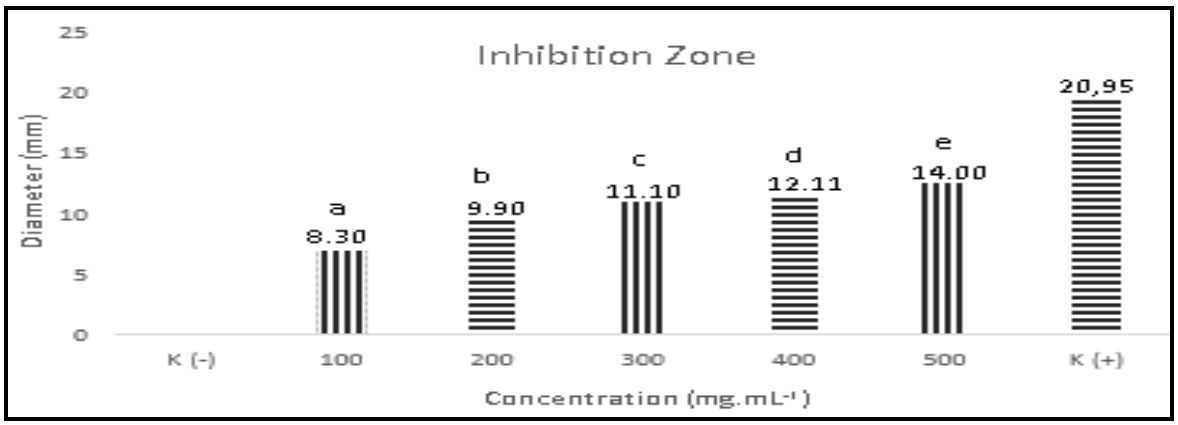

Figure 2. ntibacterial Test Results of *Plantago major* L.

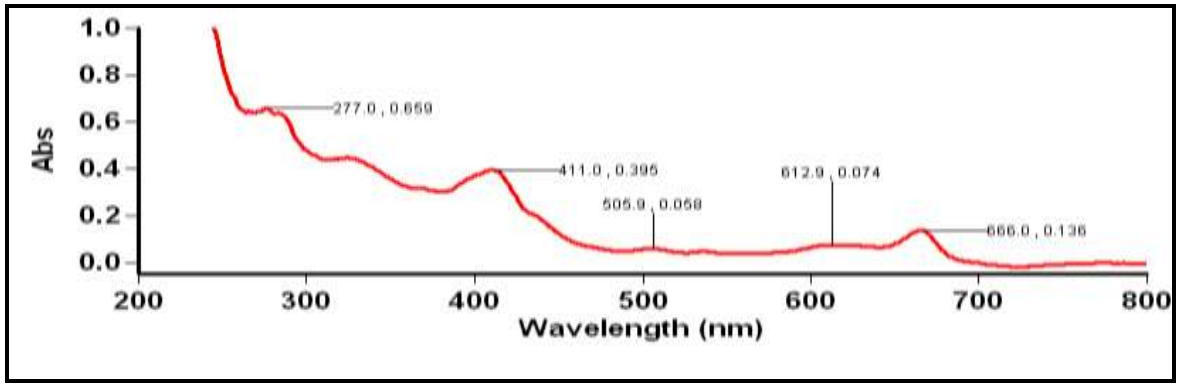

Figure 3. UV-VIS Test Result of *Plantago major* L.

Antibacterial Activity

The results of the antibacterial activity test showed that the crude extract of *Plantago major* L. had the ability to inhibit bacterial growth (Fig. 3). The inhibition of bacterial growth based on the extract dose. The higher dose, the greater diameter of the inhibition zone were formed. At the dose of 100 and 200 mg.L^{-1}, an inhibition zone is formed with medium strength, doses of 300, 400 and 500 mg.L^{-1} have a strong type of inhibition zone and the positive control using Chloramphenicol has a very strong inhibitory

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zone characteristic while the negative control using DMSO 10% does not create an inhibition zone. The provisions of the inhibitory level refer to Erlyn [28], the diameter of the inhibition zone ≥ 20 mm are included in the very strong category, 10 – 20 mm are included in the strong category, 10-5 mm has a medium category and ≤ 5 mm has a weak category.

The measurement results of the inhibition zone diameter were confirmed by the co-culture test presented in (Table 3). From the co-culture test explained that there was a decrease in the number of bacteria along with the larger dose given. This result strengthens the statement that *Plantago major* L. extract had the ability to inhibit the growth of A. *hydrophila.* Furthermore, *Plantago major* L. extract was able to significantly inhibit the growth of A. *hydrophila* ($P < 0.05$).

Table 3. Co-culture Test Result

No.	Dosage ($mg.L^{-1}$)	Colony Forming Unit ($CFU.ml^{-1}$)
1	C (+)	0
2	100	$2.10 . 10^{13}$
3	200	$1.81 . 10^{13}$
4	300	$1.23 . 10^{11}$
5	400	$1.02 . 10^{10}$
6	500	$8.6 . 10^{9}$
7	C (-)	$3.65 . 10^{18}$

Based on phytochemical, FTIR and UV-VIS test results, *Plantago major* L. extract contain some compounds such as phenols, flavonoids, saponins and tannins which are capable to inhibit the growth of A*. hydrophila*. Phenol as antibacterial has a mechanism to inhibit bacterial growth by inactivating cell membrane proteins. Furthermore, phenol will bind to proteins in the structure of the bacterial cell wall to form hydrogen bonds [29]. Hydrogen bonds will make cell wall proteins and cytoplasmic membranes damaged so that there is an imbalance between the macromolecules and ions in the cell [30,31].

Flavonoid as antibacterial work in various ways, including inhibition of DNA gyrase, inhibition of the cytoplasmic membrane and energy metabolism [32]. Flavonoids can damage the permeability of bacterial cell walls by binding to cell wall proteins so that bacterial growth will be inhibited [33]. The other flavonoid activity as an antibacterial is by binding to proteins so that it affects permeability, then it will enter into bacterial cells resulting in coagulation of proteins and causing inactivation of bacterial enzymes [34].

Saponin works by influencing the bacterial cell wall stress, this compound will bind to bacterial lipopolysaccharide which results in increased cell wall permeability and decreased surface tension of cell walls. Then saponin will enter the bacterial cell and disrupt the metabolism which causes the cell to lysis [36]. Tanin as an antibacterial will work by means of protein denaturation that prevents the metabolic process from being blocked. When the metabolic process is inhibited, growth and development of bacteria will be inhibited [36].

The antibacterial mechanism between one compound and another combined together will work synergistically and be more effective in fighting bacteria [37]. On the other hand, chloramphenicol was used as a positive control in this study because it is one of the antibiotics used to control the growth of A. *hydrophila* bacteria. Chloramphenicol is known to be able to inhibit the protein synthesis of A. hydrophila by binding to ribosome subunits so that peptide bond formation occurs [33,38].

CONCLUSION

Plantago major L. was known to have polar compounds such as phenol, flavonoid, saponin, and tannin. Phenol and flavonoid compounds contained in *Plantago major* L. are strengthened by wave detection from FTIR and UV-VIS results. *Plantago major* L. extract is known to have a significant effect on the inhibition of the growth of A. *hydrophila* bacteria significantly. In line with the result of this study, it can be suggested that further research on the antibacterial activity the plant extract with more solvent with a wider range of dosage.

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Potential of Dayak Onion (*Eleutherine palmifolia* (L) Merr) Extract As Antibacterial Against *Pseudomonas fluorescens*

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Abstract

Dayak onions (*E. palmifolia* (L) Merr) is a herb used as medicine and known to have antibacterial activity. This research aimed to evaluate antibacterial activity of Dayak onion (*Eleutherine palmifolia* (L) Merr) extract against fish pathogen *Pseudomonas fluorescens*. The method used in this study was extraction by maceration, UV-Vis spectrophotometry analysis on the extract and antibacterial activity test with Minimum Inhibitory Concentration (MIC) as well as disc diffusion test. The results of this study showed that the yield value was 8.87%. Based on UV-Vis analysis, it confirmed that extract of Dayak onion (*Eleutherine palmifolia* (L) Merr) contains predominantly flavonoids and phenols with its derivatives, that have antibacterial activity. The results of MIC and disc diffusion test showed potential antibacterial activity against *P. fluorescens,* as observed from the value of absorbance and inhibition zones that formed.

Keywords: Antibacterial, Disc diffusion, *Eleutherine palmifolia* (L) Merr, MIC, *Pseudomonas fluorescens*.

INTRODUCTION

Pseudomonas fluorescens is a gram negative bacterium that is pathogenic to fish, thus causing the disease in fish [1]. *P. fluorescens* is not only causes hemorrhage on the skin and fins, but also damages gills, kidney, and liver, which leads to fish death [2]. Infection of pathogenic bacteria such as *P. fluorescens* will cause economic losses to aquaculture industries [3].

One of the herbs that can be used against *P. fluorescens* is Dayak onion (*Eleutherine palmifolia* (L) Merr). Dayak onion grows in Borneo mountainous areas with green leaves and red bulbs like red onion [4]. The onion is known to contain alkaloids, flavonoids, phenolics, tannins, and triterpenoids, which have a function as antimicrobials [5]. This onion ethanol extract showed positive results on flavonoids, phenolics, alkaloids, saponins, and triterpenoids [6].

Other studies have previously proven that extract of Dayak onion bulbs was able to inhibit *P. fluorescens* bacteria through the Minimum Inhibitory Concentration (MIC) test [7]. The ethanol extract with a concentration of 1% was also able to inhibit the growth of *S. aureus* and with a concentration of 15% can inhibit the bacteria *T. rubrum* [8]. However, there is no study reported on the effect of the Dayak onion extract towards inhibition zone of *P. fluorescens* bacteria. Based on these facts, the aim of this research was to evaluate the antibacterial activity of Dayak onion (*Eleutherine palmifolia* (L) Merr) extract against *Pseudomonas fluorescens* bacteria.

MATERIAL AND METHOD

Extraction

Dayak onion bulbs (*Eleutherine palmifolia* (L) Merr) obtained from UPT Materia Medica Batu were extracted by maceration and the values of the yield were calculated [9]. 100 g of simplicia were mixed with 600 mL of ethanol (PA) solvent and left to stand for three days. The mixture was filtered with Whatman filter paper (No. 42). Then, the filtrate was concentrated with a vacuum rotary evaporator at 50°C until a thick extract was obtained. The yield value was determined by calculating the final weight of extract compared to the weight of the initial simplicia.

UV-Vis Spectrophotometry Analysis

UV-Vis spectrophotometry analysis was carried out by measuring the absorbance of UV wavelength spectra of the extract with a spectrophotometer [10]. The extract was transferred into the cuvet then scanned with a spectrophotometer with a resolution of 1 nm (from 200 to 800 nm). The obtained graph was analyzed for presence of active compounds.

Bacterial Culture

Pure culture of *Pseudomonas fluorescens* bacteria was obtained from the Brackishwater Aquaculture Center (BBPBAP) Jepara. Before being used for the antibacterial test, the bacterium was cultured in liquid medium [11]. The rejuvenated bacterial culture was inoculated

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using a needle loop, into *Tryptone Soya Broth* (TSB) medium. The inoculated medium was then incubated for 24 hours (37°C). After 24 hours growth, the culture was ready to be used for antibacterial testing.

Minimum Inhibitory Concentration (MIC)

The MIC test was adopted from Nordin *et al*., 2019 [12]. Preparation of the MIC test was carried out by preparing sterile test tubes according to the concentration to be used along with negative control of *P. fluorescens* and positive control containing 30 ppm chloramphenicol. The concentrated extract which has been dissolved with 10% DMSO at a predetermined concentration was transferred as much as 1 mL into a test tube containing the medium at the treatment of each concentration then [12]. Extract of Dayak onion with concentrations of 1000 ppm, 500 ppm, 100 ppm, 10 ppm, and 1 ppm were transferred into sterile test tubes. Then each tube was inoculated with 1 mL of *P. fluorescens* bacterial isolate from previous liquid medium (TSB) and incubated at 37°C. After 24 hours, the optical density of each tube was measured with a spectrophotometer with a wavelength of 600 nm. The data were analyzed to determine the MIC value.

Disc Diffusion Test

The disc diffusion test was performed according Sekar and Rashid [13] to measure the inhibition zone occurred around the disc paper that has been given Dayak onion (*Eleutherine palmifolia* (L) Merr) extract. Bacterial isolate at a density of 10^6 CFU mL^{-1} was spread on *Pseudomonas* Selective Agar Base (PSA) medium . Further, paper discs were soaked in Dayak onion extract which had dissolved using 10% DMSO to get the concentration of 1000 ppm, 500 ppm, 100 ppm, 10 ppm and 1 ppm. The discs were placed in the middle of the agar media which has been inoculated with the bacterial culture. Petri dishes were incubated for 24 hours at 37°C, and the clear zones were measured using digital calipers.

RESULT AND DISCUSSION

Extract of Dayak Onion

The extraction of Dayak onion obtained 8.87 g crude extracts. Hence, the yield was 8.87%. This showed that the secondary metabolites contained in the onion extract were dominated by the polar group. Ethanol are the best solvents that can extract almost all low molecular weight compounds such as flavonoids, alcohols, and saponins [14]. The ethanol extract has a higher extract weight than to other solvents. Also, polar solvents such as ethanol extract phenolic compounds from these plants, with more yield [15].

UV-Vis Analysis

Result of UV-Vis analysis on Dayak onion extract was presented in Figure 1. Based on the graph obtained through UV-Vis spectrophotometer it can be analyzed the absorbance values on wavelengths of 202.0, 204.1, 205.9, 208.9, 212.1, 215.1, 216.9, 222.0, 224.9, 231.0, 234.0, 237.9, 242.0, 245.0 and 367.1 nm. It is known that the value of these wavelengths is a group of flavonoids and phenols with its derivatives [16-17]. It confirmed that Dayak onion extract contains dominant compounds from flavonoids and phenols as well as its derivatives. Flavonoids are known to work as antibacterial. This compound denature proteins that will stimulate the cell destruction of bacterial cells and eventually cause lysis of bacteria [18]. The antibacterial activity can also be explained by the inhibition of bacterial metabolism and absorption of substances needed for bacterial growth [19]. Phenol compounds will disrupt the permeability of gram-negative bacterial cell membranes and interfere with the work system of bacterial metabolism [20].

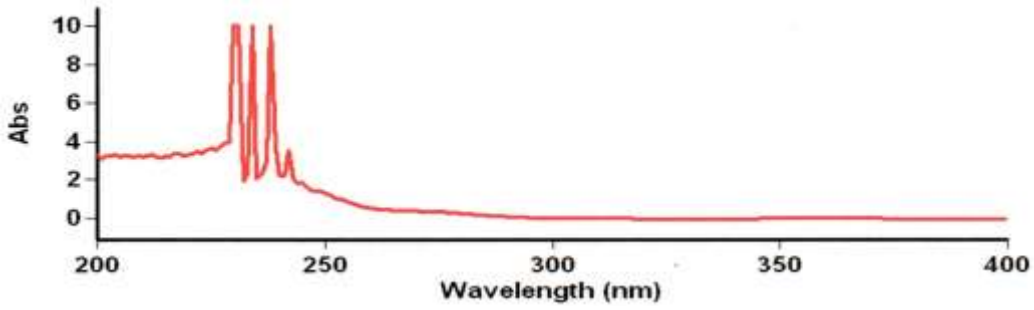

Figure 1. UV-Vis Analysis Result of Dayak Onion (*Eleutherine palmifolia* (L) Merr) Extract

Minimum Inhibitory Concentration (MIC)

MIC test was carried out to determine the minimum concentration that can inhibit bacterial growth. MIC results of Dayak onion extract on *P. fluorescens* bacteria can be seen in Table 1.

Table 1. MIC Result of Dayak Onion (*E. palmifolia* (L) Merr) Extract against *P. fluorescens*

Concentration (ppm)	Absorbance	Turbidity
1000	0.292	Quite Clear
500	0.376	Quite Clear
100	0.328	Quite Clear
10	0.192	Quite Clear
1	0.602	Turbid
Negative Control	1.161	Turbid
Positive Control	0.088	Clear

Based on the results of the MIC, it can be seen that the concentration of 10 ppm had an absorbance value lower than 100 ppm and approached the absorbance value of positive control. This shows that 10 ppm concentration of the extract can inhibit *Pseudomonas fluorescens* bacteria. The low absorbance value indicated no bacterial growth or inhibited bacterial growth [21]. The high and low concentrations of extract that causes differences in the MIC value, so it can be seen there is a presence or absence of bacterial growth [22]. Previous study stated that Dayak onion extract contained other metabolites like tannins which are derivatives of the phenolic group [23]. Phenol is an antibacterial compound that has the ability to damage bacterial cells and inhibit bacterial growth. Tannins, as its derivates can bind with lipoteichoic on the surface of bacteria [24]. Tannins can agglomerate bacterial cytoplasmic cells so that the metabolism of bacteria will be disrupted [25].

Disc Diffusion Test

The results of the disc diffusion test, showed the clear zone (inhibition zone) formed by different bacterial inhibition categories, such as very strong, strong, medium and weak [26], which can be seen in Table 2.

Table 2. Disc Diffusion Test Result of Dayak Onion (*E. palmifolia* (L) Merr) Extract against *P. fluorescens*

Concentration (ppm)	Clear Zone Diameter (mm)	Inhibitory Categorial
1000	13.04 ± 0.21	Strong
500	11.37 ± 0.52	Strong
100	10.79 ± 0.32	Strong
10	9.23 ± 0.54	Medium
1	6.10 ± 0.04	Medium

In these results, it can be seen that the onion extract has antibacterial activity. Where the clear zone formed indicated inhibition of bacterial growth [27]. It showed that the higher the concentration of extract, the greater the clear zone formed, with the largest clear zone at a concentration of 1000 ppm, which is 13.04 ± 0.21 mm. Other studies previously showed that Dayak onion extract was able to inhibit pathogenic bacteria such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Shigella* sp. and *Bacillus cereus*, with concentrations of extract was 10 ppm [28]. This depends on the content of secondary metabolites that contained in that onion. Based on this study, the onion extract contains flavonoids and phenols compound along with its derivatives which are known to have antibacterial activity.

Flavonoids as antibacterial have three ways of working, the first by inhibiting nucleic acid synthesis, the second is by inhibiting cytoplasmic membrane function and the third by inhibiting energy metabolism [29,30]. Phenol can inhibit bacteria by destroying the plasma membrane. Where the damage of that cell membranes can prevent food or nutrients needed by bacteria to produce energy, resulting in inhibition of growth or even death [31]. In general, the work of antibacterial compounds cause membrane permeability disorder that lead to leakage of the cytoplasmic membrane. Cytoplasmic membranes can become damaged and functionally deactivated when bacteria are exposed to antibacterial agents. So the material of the intracellular will leak out that results in the damage on large molecules in bacteria [32].

CONCLUSION

The extract of Dayak onion (*Eleutherine palmifolia* (L) Merr) showed the presence of antibacterial compound such as flavonoids and phenols along with its derivatives. The crude extract inhibited the growth of *Pseudomons fluorescens* bacteria, where the MIC results that obtained was at 10 ppm of concentration. Based on the disc diffusion test, it was found that the extract concentration will affect the antibacterial activity in inhibition.

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Growth and Development of *Rhyzopertha dominica* Fabricius (Coleoptera: Bostrichidae) on White, Red and Black Rice

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Abstract

Rhyzopertha dominica is a polyphagous stored pest to many grains including rice. *R. dominica* can easily spread from one place to another to infest stored products. The aim of this research was to know the influence of three grains types with different weight to *R. dominica* growth, development, net reproduction rate and gross reproduction rate on red, black and white rice. The research was conducted in Pest Laboratory, Department of Plant Pests and Diseases, Faculty of Agriculture, University of Brawijaya Malang. The research result showed that there was an interaction among three grains types with different weight to the number of *R. dominica* larval, pupal, and newly adult emerged, while the number of eggs, weight of newly adult emerged, the reduction percentage of grain and phase of the egg, larval, pupal and the life cycle period of *R. dominica* were affected by different grains types factor. The R_0 value showed that on the next generation, number of female was increase 1.25 times from the previous generation on white rice, while on red and black rice the population was increase up to 2.63 and 2.49 times from the previous generation.

Keywords: Growth and Development, Reproduction Rate, Rice*, Rhyzopertha dominica.*

INTRODUCTION

There are many kinds of stored products that are stored in warehouse. Rice is one of the products that are stored in the warehouse. Based on the color of the rice, there were several types of rice are known in Indonesia as white, black, red and glutinous rice. Rice consist of various nutritional content such as carbohydrates, proteins, fats, vitamins, minerals and other components. Yield losses of stored rice in the warehouse are caused by the presence of pests [1]. Pests of stored grain such as Lesser Grain Borer (*Rhyzopertha dominica* F) is one of the pest which are classified as primary pests that can be invested on various types of seeds such as wheat, corn, rice and other cereal crops. The weight of rice grain is decrease due to the damage caused by larval and adult of *R. dominica* [2]. Based on previous study [3], using six varieties of rice milled (IR-64, Cibogo, Ciherang, Membrano, Sembada, and Intani-2) which showed that the number of eggs laid by imago *R. dominica* females, the number of new progeny (F1) and a decrease in weight loss on membrano rice variety was lower followed by the Ciherang rice variety, IR-64, Cibogo, Sembada and Intani-2. Based on the index of susceptibility, Membrano is the resistant, while Ciherang, IR-64, Sembada and Cibogo are the moderately resistant rice varieties, and Intani-2 is the highly susceptible. The aims of this research is to know the effect of three grains types with different weight to *R. dominica* growth, development, net reproduction rate and gross reproduction rate on red, black and white rice. Therefore, this study can provide information about the chance of *R. dominica* growth and development to invest on white, red and black rice as well as the net reproduction rate of *R. dominica* in the next generation.

MATERIAL AND METHOD

The research was conducted in Pest Laboratory, Department of Plant Pest and Diseases, Faculty of Agriculture, University of Brawijaya, Malang from October 2017 to September 2018. The tools that used in this study were hand counters, scales, scissors, brush sizes 00 and 1, rubber band, trays, glass tubes d = 6.5 and t = 9, digital cameras, refrigerator, thermohygrometer, Petri dish d = 10 and t = 1.5, Olympus S2X7 + Camera DP 26 microscope. The materials that used in the study were white rice of Ciherang variety, red rice, black rice, white chiffon type fabric, paper label.

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Research Preparation

Rearing of Insects

R. dominica insects were obtained from Biotrop Entomology Laboratory, Bogor. Propagation of *R. dominica* was carried out by following the method [4]. About 100 pairs of *R. dominica* were infested on glass tubes t = 28cm d = 20cm containing 500 g of white rice of Pandanwangi variety. The glass tube was covered with white chiffon and labeled then stored in a room with a temperature of 27 ± 2°C and humidity of 65 ± 5%. Adult of *R. dominica* infested for one week. The infested rice was left until newly adult emerge. The first progeny (F1) *R. dominica* was used in this study.

Preparation of the Research Medium

The research was carried out using Ciherang variety of white, red and black rice which obtained from Kanigoro Village, Pagelaran, Malang, East Java, Indonesia. Sterilization of red, black and white rice was carried out following the method [4] using a refrigerator (freezer) at approximately -15°C for one week. The rice was transferred to the refrigerator at 5°C for one week to protect the rice from re-infestation of insects and mites. Rice was placed at 27 ± 2°C for two weeks before the research started.

Physical and Chemical Analysis

Physical and chemical analysis of the rice consists of hardness feed test, proximate and phenol test. The hardness test of rice was carried out using a food texture measuring tool, namely Universal Texture Analyzer. Hardness test was carried out to determine the hardness level of rice before the research used. The proximate test was carried out to determine the value of carbohydrate, protein, fat, water and ash content in each type of rice before being used in this study. The phenol test was carried out to know the phenol content in each type of rice which used on this research by Spectrophotometry analysis method. The result of physical and chemical analysis showed in Table 1.

Laboratory Temperature and Humidity

The temperature and humidity in the laboratory were daily observed using Thermohygrometer at 06.00 am, 12.00 pm and 06.00 pm.

Table 1. Physiscal and Chemical Parameter of Rice

Type of Rice	Protein (%)	Fat (%)	Water (%)	Ash (%)	Carbohydrate (%)	Hardness of Rice (N)
Red	7.61	2.18	11.68	1.21	77.32	35.98
Black	7.89	2.64	11.65	1.64	76.18	63.80
White	7.35	0.33	12.31	0.36	79.65	71.87

Research Implementation

Growth and Development Research of R. dominica

The study was conducted using no choice method on white, red and black rice. The study was arranged by Completely Randomized Design consisting of two factors and six replication. The first factor was the type of rice (white, red and black rice) and the second factor was the weight of the feed (20, 30 and 40 g). Infestation of *R. dominica* was done by infesting 15 pairs into each weight treatment, then the tube was covered by chiffon. Adult of *R. dominica* was infested for seven days. The observed variables were:

a. Mortality of *R. Dominica* adults. The observation was done by calculating the number of adults *R. dominica* that lived and died after seven days of early infestation.
b. The Number of eggs, Larval, pupal and F1 Progeny emerged *R. dominica*. The egg that produced by 15 pairs of adults *R. dominica* were observed on microscope. The calculated eggs are re-put into the rice to be observed for the number of larval, pupal, and F1 progeny. The F1 Progeny that appears was released every day until there is no new adult (F1) emerged, then calculated and observed the sex ratio.
c. Calculation of the percentage of weight reduction by using a formula [5] :

$$\textbf{Weight Loss}\ (\%) = \frac{(\textbf{Wu x Nd}) - (\textbf{Wd x Nu})}{\textbf{Wu x}\ (\textbf{Nd} + \textbf{Nu})} \times 100\%$$

Description:
Wu = weight of whole seeds
Wd = weight of damaged seeds
Nu = number of whole seeds
Nd = number of damaged seeds

d. The calculation of susceptibility index was followed Dobie index [6], where in the Dobie index was based on the number of the first progeny emerged (F1) *R. dominica* and the median development time:

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$$\mathbf{IK} = \frac{[\mathbf{Loge(total\ F1\ Progeny\ emerged)}]}{\mathbf{Median\ development\ time}} \times \mathbf{100}\%$$

The index of susceptibility (IK) consists of four categories:

IK 0 to 3 = Resistant
IK 4 to 7 = Moderately resistant
IK 8 to 10 = Susceptible
IK ≥ 11 = Highly susceptible

e. Calculation of the weight of new adult emerged *R. dominica* was carried out by sampling 10 individuals per treatment. So that the average weight of new *R. dominica* in each treatment was obtained [7].

The Observation of the egg longevity was done by taking a sample of 10 eggs placed on the same day on each type of feed. Eggs were observed daily until hatched into larval. The observation of the Larval longevity was carried out by taking samples of 10 Larval that released on the same day. These larvae were placed on bottles d = 3 t = 3 and infested on each feed treatment. Observations were made from the first time the Larvae released until the Larval turned into pupal. The observation of the pupal longevity was done by observing the holes in each grain treatment. Observations were carried out until the pupa changed to the F1 Progeny emerged. New male and female of *R. dominica* were mated to find out the time of first egg laid. The life cycle of *R. dominica* can be determined by calculating the required time of *R. dominica* in each treatment since the egg laid until the F1 progeny laid an egg for the first time. Observations were made using six replications.

Observation of the life table is to know the rate of net reproduction and the gross reproduction rate of *R. dominica* on white, red and black rice. Observations were made by taking 100 eggs placed on the same day by adults *R. dominica* in each treatment. Observations were made by recording the number of eggs that had succeeded in hatching into Larval, then the Larval that succeeded to pupal, and the pupal that had successfully became new F1 Progeny *R. dominica* (lx). Observations were made using six replications. The observation of the mean number of eggs laid on each treatment is done to determine the number of offspring produced (mx).

Data Analysis

The data were analyzed using analysis of variance (ANOVA) at the 95% confidence level, and followed by LSD's test at 95%. Data of life table was analyzed and arranged in cohort. Based on data that compiled in the life table, the gross reproduction rate (GRR) is calculated from data on the average number of eggs laid in each treatment (mx) or with the formula ∑mx and the net reproduction rate or Net Reproductive Rate (R_0) derived from the total number of female adults or with the ∑lxmx formula [8,9].

RESULTS

Mortality of Imago *R. dominica* After Seven Days of Early Infestation

Based on analysis result, the interaction among three types of rice with different weights did not significantly affect mortality of adult *R. dominica* after seven days of early infestation. The means mortality of *R. dominica* after seven days of early infestation on three types of rice with different weights showed in Table 2.

Table 2. The Means Mortality of *R. dominica* After Seven Days of Early Infestation in Three Types of Rice with Different Weights

Interaction (Rice and Weight)	Mortality of adults *R. dominica* After Seven Days of Early Infestation (%) (x̄ ± SD)
Red Rice 20 g	7.78± 7.20
Red Rice 30 g	6.67± 7.30
Red Rice 40 g	5.56 ±5.02
Black Rice 20 g	3.33± 2.98
Black Rice 30 g	3.89± 3.28
Black Rice 40 g	7.22± 6.47
White Rice 20 g	2.78± 1.36
White Rice 30 g	6.11± 5.74
White Rice 40 g	5.56± 5.44
LSD's 5%	ns

Notes: Data is transformed by the formula √ (x + 0.5) for analysis purposes; SD is a Standard Deviation; the sign (tn) shows there is not significant.

Number of Eggs Laid by *R. dominica* After Seven Days of Early Infestation

Based on analysis result showed, there was no interaction among the three types of rice with different weights for the mean number of eggs laid by *R. dominica* after seven days of early infestation, but there was an influence on each factor on the mean number of eggs laid by *R. dominica* adults. The mean number of *R. dominica* eggs with the influence of different types of rice was presented in Table 3.

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Table 3. The Mean Number of Eggs of *R. dominica* With The Effect of Different Types of Rice

Type of Rice	Eggs of *R. dominica*(x̄ ± SD)
Red	262.94 ± 80.02 b
Black	249.00 ± 43.81 ab
White	224.00 ± 87.00 a
LSD's 5%	*

Notes: A number which followed by the same letter showed the results did not differ markedly on the advanced test using the Tukey's test on 5% faults levels; SD is a Standard Deviation; the sign (*) shown there is a significant.

Table 4. The Means Number of Eggs of *R. dominica* With Effect of Different Feed Weights

Weight of Rice	Eggs of*R. dominica*(x̄ ± SD)
20 gram	173.11 ± 42.54 a
30 gram	251.72 ± 12.54 b
40 gram	311.11 ± 26.77 c
LSD's 5%	*

Notes: A number which followed by different letters showed significantly different results on the advanced test using the Tukey's test on 5% fault levels; SD is a Standard Deviation; The sign (*) shownthat there is a significant.

In Table 3 showed a significantdifference in the type of rice to the average number of eggs placed by *R. dominica* after seven days of early infestation. The average number of eggs placed by *R. dominica* after seven days of early infestation on red rice was higher at 262.94 grains compared to black rice (249.00 grains) and white rice (224.00 grains). The means number of eggs *R. dominica* with the influence of different feed weights is presented in Table 4. Table 4 shows that there is a significant difference in different weight of rice to average number of eggs laid by imago *R. dominica* after seven days early infestations.

Amount of Larval, Pupal and F1 Progeny Emerged of *R. dominica*

Based on analysis result showed that average range of the number of Larval, pupal and F1 progeny emerged of *R. dominica* pointed out that there were an interactions among the three types of rice with different weight. The mean number of Larval, pupal and F1 progeny emerged of *R. dominica* with the influence of three types of rice with different weight was presented in Table 5.

Table 5 showed the number of Larval, pupal and F1 progeny emerged of *R. dominica* on white rice were significantly different red and black rice. Based on the correlation test showed that there was a positive correlation in the fat content in rice by the number of Larval (r = 0.806; p = 0.009), pupa (r = 0.836; p = 0.005) and adult (F1) *R. dominica* (r = 0.805; p = 0.009). This was suggested that the higher the fat content in rice, the higher the number of Larval, pupal and F1 progeny emerged of *R. dominica*. Conversely, the lower the fat content in rice, the lower number of Larval, pupal and adults emerged.

Table 5. The Mean number of Larval, Pupal and F1 Progeny Emerged of *R. dominica*with The Influence of Three Types of Rice with Different Weight

Interaction (Rice and Weight)	Larval (x̄ ± SD)	Pupal[1] (x̄ ± SD)	F1 progeny *R. dominica* (x̄ ± SD)
Red Rice 20 g	156.50 ± 19.18 bc	135.67 ± 22.85 b	122.83 ± 22.19 b
Red Rice 30 g	200.83 ± 55.73 cd	174.83 ± 55.96 bc	162.17 ± 54.82 bc
Red Rice 40 g	308.33 ± 42.57 e	289.83 ± 45.41 d	276.83 ± 43.96 d
Black Rice 20 g	162.67 ± 35.32 bc	147.00 ± 33.23 bc	107.17 ± 50.08 b
Black Rice 30 g	206.00 ± 37.27 cd	173.00 ± 20.66 bc	164.17 ± 13.89 bc
Black Rice 40 g	264.17 ± 25.92 de	222.67 ± 42.99 c	210.83 ± 39.46 c
White Rice 20 g	57.00 ± 25.28 a	26.33 ± 2.58 a	11.50 ± 2.95 a
White Rice 30 g	88.83 ± 57.66 ab	29.33 ± 3.88 a	18.33 ± 5.32 a
White Rice 40 g	77.17 ± 16.63 a	38.83 ± 5.98 a	27.33 ± 6.41 a
LSD's 5%	*	*	*

Notes: A number which followed by the same letter in the same column shows the results did not differ markedly on the advanced test using the Tukey's test on 5% fault levels. 1) Data is transformed by the formula √ (x + 0.5) for analysis purposes; SD is the Standard Deviation; The sign (*) shows that there is a significant.

Sex Ratio *R. dominica*

Based on analysis result, it showed that the sex ratio of the female are more compared to the sex ratio of male adults. The sex ratio of *R. dominica* was presented in Table 6.

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Table 6. The Sex Ratio of *R. dominica*

Interaction (Rice and Weight)	Male Adults ($\bar{x}$ ± SD)	Female Adults ($\bar{x}$ ± SD)	Sex Ratio (Male : Female)
Red Rice 20 g	54.00 ± 10.86	70.50 ± 13.10	1:1.31
Red Rice 30 g	75.00 ± 25.87	87.17 ± 29.78	1:1.16
Red Rice 40 g	127.00 ± 14.63	149.83 ± 33.77	1:1.18
Black Rice 20 g	45.17 ± 29.46	53.17 ± 33.72	1:1.18
Black Rice 30 g	77.50 ± 12.58	86.67 ± 6.15	1:1.12
Black Rice 40 g	99.83 ± 18.35	111.00 ± 21.93	1:1.11
White Rice 20 g	5.50 ± 1.38	6.00 ± 2.45	1:1.09
White Rice 30 g	7.83 ± 2.48	10.50 ± 3.39	1:1.34
White Rice 40 g	12.00 ± 2.97	15.33 ± 3.98	1:1.28

Notes: the SD is the Standard Deviation.

The Weight of F1 Progeny Emerged of *R. dominica*

The interaction among these three types of rice with different weight has no effect against the weight of F1 progeny emerged of *R. dominica*. The mean weight of F1 progeny emerged of *R. dominica* with an influence of different rice types was presented in Table 7.

Table 7. The Mean Weight of F1 Progeny Emerged of *R. dominica* with an Influence of Different Types of Rice

Type of Rice	Weight of F1 Progeny *R. dominica* ($\bar{x}$ ± SD)
Red	0.0013 ± 0.0001 a
Black	0.0018 ± 0.0003 b
White	0.0011 ± 0.0000 a
LSD's 5%	*

Notes: a number which followed by the same letter showed the results did not differ markedly on the advanced test using the Tukey's test on 5% faul levels; SD is the Standard Deviation; sign (*) shows that there is a significant.

In Table 7, it can be seen that the mean weight of F1 progeny emerged of *R. dominica* was influenced by different types of rice. The mean weight of adult on black rice was higher i.e. 0.0018 mg compared to the mean weight of the adults on the red and white rice (0.0013 mg and 0.0011 mg). Based on the correlation test showed a positive correlation (r = 0.763; p = 0.017) on fat content Rice to the weight of the new adults *R. dominica*. This was suggested that the higher the fat content in rice, the new adults *R. dominica* will get heavier. Conversely, the lower the fat content in rice, the new adults *R. dominica* will get lighter.

Egg, Larval, Pupal Longevity and Life Cycle Period of *R. dominica*

The research was conducted on the conditions of the laboratory with a mean temperature of 27°C and humidity 61%. The results of the analysis of variance showed that the interaction among the three types of rice with different weight has no effect against egg, larval, pupal longevity and the life cycle of *R. dominica*. The mean longevity of egg, Larval, pupal and the life cycle of *R. dominica* with an influence of different types of rice are presented in Table 8.

Table 8 showed that longevity of egg, larval, pupal and the life cycle of *R. dominica* was influenced by different types of rice. Average longevity of egg, Larval, pupa and the life cycle of *R. dominica* on white rice are longer, i.e. 4.48 days; 29.27 days; 10.06 days; and 52.04 days compared to black and red rice. Physical and chemical characters of feed were suspected to be the factors that cause the differences of the longevity of Larval, pupa and life cycle of *R. dominica*. It is supported by the correlation test which shows that there is a positive correlation between the hardness of feed, the moisture content and the content of carbohydrates in rice to the longevity of eggs (r = 0.773; p = 0.015; r = 0.884; p = 0.002), Larval (r = 0.944; p = 0.000; r = 0.902 ; p = 0.001), pupal (r = 0.969; p = 0.000; r = 0.997; p = 0.000) and the life cycle of *R. dominica* (r = 0.993; p = 0.000; r = 0.973; p = 0.000).

Weight Loss and Sensitivity Index Feed

The results of the analysis of variance showed that the interaction among the three types of rice with different weight has no effect to a weight loss of feed due to the infestation of *R. dominica*. The results of the analysis of the calculation method according to the index of susceptibility of Dobbie showed that different type of rice will affect the category ofindex of susceptibility. The mean of weight loss and categories of index susceptibility of feed were presented in Table 9.

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Table 8. The Means Longevity of Egg, Larval, Pupa and The Life Cycle of *R. dominica* with An Influence of Different Types of Rice

Type of Rice	Egg (days) (x̄ ± SD)	Larval (days) (x̄ ± SD)	Pupal (days) (x̄ ± SD)	Life Cycle (days) (x̄ ± SD)
Red	3.23 ± 0.28 a	27.14 ± 0.57 a	7.75 ± 0.10 a	46.15 ± 0.42 b
Black	3.56 ± 0.38 a	26.04 ± 0.81 a	7.51 ± 0.03 a	44.09 ± 0.42 a
White	4.48 ± 0.05 b	29.27 ± 0.07 b	10.06 ± 0.10 b	52.04 ± 0.19 c
LSD's 5%	*	*	*	*

Notes: A number that is followed by the same letter in the same column shows the results did not differ markedly based on Tukey's test on 5% fault levels; SD is the Standard Deviation; sign (*) shows that there is a real difference.

Table 9 showed that the different types of rice affected on the feed weight loss due to the infestation of *R. dominica*. Feed weight loss on white rice is lower compared to red and black rice. This was allegedly because the number of Larval and first progeny emerged (F1) on white rice is lower compared to the red and black rice, so the feeding activity of *R. Dominica* in Larval and adults phase on white rice lower than on red and black rice. Based on the correlation test showed that there was a positive correlation between the weight loss to the number of Larval (r = 0.819, p = 0.007) and imago *R. dominica* (r = 0.813; p = 0.008). This was showed that the higher number of Larval and new adult *R. dominica,* the higher weight loss. Conversely, the lower number of Larval and new adult *R. dominica*, the lower weight loss. Table 8 showed that the red and black rice belong to the same susceptibility category that was 'Moderately Resistant', while white rice was belong to 'resistant'.

Table 9. The Means Weight Loss and Categories of Index Susceptibility of Feed

Type of Rice	Weight Loss (x̄ ± SD)	Index Susceptibility	Category Index Susceptibility
Red	4.23 ± 0.63 b	3.60	Moderately Resistant
Black	5.24 ± 0.52 b	3.63	Moderately Resistant
White	0.74 ± 0.06 a	1.54	Resistant
LSD's 5%	*		

Notes: A number which followed by the same letter showed the results did not significantly different based on Tukey's test on 5% fault levels: SD is the Standard Deviation; sign (*) indicates the presence of a real difference; Provision of Index Susceptibility value consist of four categories; where resistant (0 to 3), moderately resistant (4 to 7), susceptible (8 to 10), and highly susceptible (more than 11).

The Life Table of *R. dominica*

Based on the life table, it was showed that the highest percentage of the die individuals (dx) occured on the eggs and larval phase. Based on the method of Price [10] gross reproduction rate (GRR) and net reproduction rate *R. dominica* can be calculated based on the amount of the value of mx and lxmx on cohort table. The value of gross reproduction rate and net reproductive rate *R. dominica* in three types of rice are presented in Table 10.

The value of the GRR in Table 10 indicates that *R. dominica* was able to produce offspring of 8.15 individuals per parent per generation on red rice, 8.12 individuals per parent per generation on black rice, and 8.53 individuals parent per generation on white rice. In Table 10, it can be seen that the value of R_0 produced by female *R. dominica* in white, red and black rice were: 1.25; 2.63; and 2.49 per individual/adult/generation. It showed that the population could rise up to 1.25 times more from previous generation on white rice, up to 2.63 and 2.49 times more on red and black rice.

Table 10. The Value of Gross Reproduction Rate and Net Reproductive Rate of *R. dominica* on White, Red and Black Rice

Type of Rice	GRR	R_0
Red	8.15 ± 2.96	2.63 ± 0.80
Black	8.12 ± 1.25	2.49 ± 0.44
White	8.53 ± 0.39	1.25 ± 0.15

Notes: GRR (*Gross Reproduction Rate*); R_0 (*Net Reproductive Rate*).

DISCUSSION

Based on research results for eggs, Larval, pupal, new imago in first progeny (F1) and first progeny's (F1) weight, R. *dominica,* for different type of rice in several weight showed that the different type of rice is more influential for first progeny (F1) and first progeny's (F1) weight *R. dominica.* Based on correlation test, there was a relationship between physical and chemical in feed matters. There are factors that influence insect's

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growth and its development, such as feeding (nutrition, texture, water content), climatic condition (temperature, humidity, illumination), natural enemies' condition (predator, parasitoid, pathogen), and human activities [11].

Insects used protein and glycogen as energy. Protein affects reproductive performance of insect directly. Stored protein of insect affects to the number of eggs, new adults emerged and size of adults. Insect with high-protein feed requirement will utilize the availability of these compounds for tissue formation, so that the Larval can reach the final instar stage more quickly. Protein provides basic substances for the formation of Larval body tissues that are used to pass the instar stage during its development, while carbohydrates tend to act more as energy sources. The nutrients received by *R. dominica* Larval not only affect the growth, development and survival of Larval, but also the ability to survive pupal to become imago *R. dominica* [12].

Based on research on the phase of the egg, Larval, pupal and the life cycle period of *R. dominica* in white rice was longer than the other treatments. The nutrient content in feed, especially carbohydrates, affects the life span of insects [13-15]. Nutrition is one of the important factors that affect the life of insects such as the process of growth, breeding, reproduction and fitness of the immune system. The growth and reproduction of insects is strongly influenced by nutrients, which is obtained during the Larval and adult insects. Larval requires balanced amounts of protein, carbohydrates, fats, vitamins, minerals, and water for optimal growth and development [16].

Based on research results, it found that the number of sex ratio of a population composition is not consistent though insect sex ratio generally balanced [17]. Activities during the Larval phase and imago *R. dominica* which feeding from the seed until the remaining outer layer, only causes the feed to become severely decreased. A female adult of *R. dominica* laid eggs outside the seeds or in the bottom of the treatment bottle. The incubation period that has been completed, causes the Larval to come out of the egg by making a circular hole on the egg surface. The newly hatched Larval is whitish with a brownish head. After some time, the Larval enter the grain through the soft part of the feed [18].

The Larval feed the inside of the seeds throughout their lives to complete the phase and turn into pupal in the seeds. Pupa will be inside the seeds until they turn into the new *R. dominica* image. The new adult *R. dominica* will eat the inside of the seeds until it can get out of the seeds. Larval and adult *R. dominica* when feeding will produce large amounts of excreta with impurities that resemble pellet granules. Larval emit pellet-like granules when infesting feed, causing odors that make it easy to detect *R. dominica* infestations [19].

Based on the index of susceptibility value for the treatment of three different types of rice with different weights, showed that white rice was catagorized as resistant, while black rice and brown rice were rather resistant category. A higher diversity index indicates higher susceptible varieties. Varieties with a higher susceptibility index produce maximum progenies and vice versa, varieties with a lower susceptibility index produce a minimum number of progenies [20].

Population growth is very dependent on the female adults that are able to survive as well as the number of eggs that were successfully laid. Based on these factors, it can be done on population size predictions for the next generation of a population insect that had previously been known. The calculation of the reproduction rate is required to infer the population growth of an organism [10]. The gross reproduction rate is the average number of offspring per female carriers produced by the individual whose life reaches a maximum age. Meanwhile, the net reproduction rate is the value of the average abundance of offspring produced by individual females every generation after taking into account death or life chance (lx) [21].

This study showed that the GRR value of white rice is higher compared to GRR in red and black rice. But the value of R_0 for white rice is lower compared to brown rice and black rice. The high mortality rate occured in the egg phase and the Larval of the first instar *R. dominica* recorded during the wet season (November-December 2014), were caused by several factors: 1) the appearance of parasitoid *R. dominica* Larval, such as: *Anisopteromalus calandrae* (Howard) and *Theocolax elegans* (Westwood) (Hymenoptera: Pteromalidae), 2) predatory mite infestation of Newport *Ventricosus pyemotes* (Acari: Pyemotidae)

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in eggs and Larval, 3) low temperature conditions when the study was conducted. The low temperature causes the eggs laid by *R. dominica* slightly and cannot hatch due to inappropriate conditions, so that the number of Larval needed is not sufficient for further biological studies of *R. dominica* during winter [22].

CONCLUSION

Growth and development of *R. dominica* was not influenced by interactions between the types of rice and different weight, but an influential factor was the different types of rice. The reproduction rate of *R. dominica* on red, black and white rice showed the population will tend to increase in the next generation.

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The Effect of Biogas Waste Doses (*Sludge*) of Cow Manure on the Growth and the Yield of Eggplant (*Solanum Melongena* L.)

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Abstract

The growth and the yield of eggplant plants are strongly influenced by the condition of the soil, and any damage to soil quality can cause unproductive plants according to its potential results. The addition of organic material in the form of biogas waste into the soil is expected to increase the yield of eggplant. This experiment aims at determining the best dose of biogas waste (sludge) to increase eggplant production. The experiment was carried out in Jember Lor village, Patrang, Jember, East Java with an altitude of ± 89 meters above sea level (m asl). The experiment used a completely randomized design method (CRD) with 4 treatments, treatment L0: 0kg waste plant^{-1}; L1: 0.6 kg of waste plant^{-1}; L2: 1.2kg of waste plants $^{-1}$ and L3: 2.2 kg of waste plants^{-1}. Each treatment was repeated 6 times with a total of 24 plants. Based on the results of the study, there was a very significant effect of sludge on variables of plant height, flowering age, harvesting age, fruit diameter, fruit length, and fruit weight. Sludge does not show a significant difference in plant diameter variables. The best treatment to have reached the plant height, fruiting age, harvesting age, fruit diameter, fruit length and fruit weight was the treatment of using a sludge with the dose of 1.2 kg polybag^{-1} (L2) with a height of 102.8 cm, different ages of 30.83 HST , harvest age of 56.73 HST, fruit diameter of 35.93 mm, fruit length of 17.95 cm and fruit weight of crop reached 1.36 kg (37.61 tons ha^{-1}).

Keywords: biogas waste, cow dung, eggplant, sludge.

INTRODUCTION

Eggplant (*Solanum melongena L.*) is a tropic native plan originating from the Asian continent, especially India and Burma. Eggplant is a vegetable plant that includes in the eggplant-family (Solanaceae). Eggplants become everyone's favorite vegetable, both as fresh vegetables and processed into various types of cuisine [1]. Eggplant contains various kinds of nutrition. Every 100 grams of fresh eggplant contains 24 g of calories, 1.1 g of protein, 0.2 g of fat, 5.5 g of carbohydrates, 15 mg of calcium, 37.0 mg of phosphorus, 0.4 mg of iron, 4.0 mg of Vitamin A, 5 mg of Vitamin C, 0.04 mg vitamin B1, and 92.7 g of water [2].

The demand for eggplants continues to increase, but the demand escalation is not accompanied by an increase in the amount of production. One of them is caused by the low productivity of eggplant. Indonesia has an average production of eggplant ranging from 9.38 - 10.95 tons hectare^{-1}. Whereas, the one-hectare area actually can produce 30 tons of eggplant [3]. Some damages to soil quality cause more unproductive plants according to the potential results.

The use of excessive inorganic fertilizers without being followed by organic fertilizers causes damage to the soil. Damage to the soil resulted in the inefficiency fertilizer due to the loss of fertilizer from the soil through erosion, leaching and binding by other compounds. Adding green manure and organic fertilizer can be made as an effort to reduce the loss of fertilizer [4].

Biogas waste is an appropriate organic material from daily livestock waste for sustainable, environmentally friendly and pollution-free agricultural production. Biogas waste can increase the agricultural production due to nutrient content, enzymes and growth hormones contained in it. Biogas waste fertilizer has the same benefits as manure to improve soil structure and provide nutrients needed by plants [5]. Organic manures play a direct role in soil fertility, microbial population, improves plant growth by providing micro and macronutrients in available form, which eventually increased productivity [6].

Provision of biogas organic fertilizer from cow manure with a concentration of 20 mL.L^{-1} of water plot^{-1} can increase the yield of sweet corn 0.8 ton ha^{-1} [7]. Addition of 300 mL biogas wastes plants^{-1} can increase plant height, a number of leaves and diameter of oil palm plant seedlings.

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Utilization of cow biogas waste to increase crop productivity has not been done. The purpose of this study was to determine the effect of biogas waste dose (*sludge*) on the growth and production of eggplant plants.

MATERIAL AND METHOD

The trial was conducted at the Green House in Jember Lor, Partang, Jember with an altitude of ± 89 masl and starting from April-November 2018. This experiment used a completely randomized design (CRD) method with 4 treatments. Treatment of L0: 0kg of waste polybag^{-1}; L1: 0.6 kg of waste polybag^{-1}; L2: 1.2kg of waste polybag^{-1} and L3: 2.2 kg of waste polybag^{-1}. Each treatment was repeated as many as 6 replications with a total of 24 plants.

Extraction and Drying of Biogas Waste

The used biogas waste came from biogas reactors in Kemungsari Lor village, Panti, Jember. The taken waste was then dried until the water content ranges from 10-40% to avoid ignoring the elements N and P.

Media Preparation

The planting media used were the land originating from Bintoro village, Patrang, Jember. The planting media was in the form of land that had dried up the wind, weighing in as much as 10kg and put into polybags size 50 x 50. The distance between polybags used was 60cm x 60cm. After the polybag was prepared, then fertilization was carried out using biogas waste on seven days before planting in accordance with the treatments.

Preliminary Analysis of Soil and Biogas Waste

The analysis was carried out by taking soil samples and biogas waste which would be used in the study, then, drying the air and sieving using a 2 mm diameter sieve. While the biogas waste samples were dried until the water content was below 40%. Examples of soil and waste that have been dried were then determined for total N-nutrient levels, total P, total K, organ-C, and pH. N-total was determined by Kjeldahl distillation, P-total was determined using the P-Olsen method, K-total was determined using the 25% HCl Extract method while organic C was carried out by Walkley and Black method and pH using H_2O method (1: 2.5).

Seedling

Seedlings nurseries were planted in 5x10cm polybags. The planting media used for the nursery process was compost. Each polybag was filled with one seed. The nursery was carried out for ± 28 days until it was ready to move with the characteristics of leaves formed as many as 4-6 leaflets.

Planting and Maintaining

The Eggplant seeds that were 28 days old done trough transferring those into a 50x50 cm polybag. Each polybag was filled with one seed with a distance of 60X60 cm polybag. Eggplant plants were planted for 75 days and were observed every 10 days for growth variables. Maintenance includes watering, weeding, and prevention as well as handling physical pest and plant organisms (OPT).

Analysis of Soil Samples After

The samples of soil were analyzed for organic carbon (C-Organic) and cation exchange capacity (CEC) at 21 days after planting. The analysis was carried out by taking soil samples at each polybag then compositing. Then dry the air and sift using a 2 mm diameter sieve. The example then set the CEC and C-organic values.

Data processing

The data obtained was carried out by the analysis of variance (ANOVA) and then followed by a Duncan multiple range test (DMRT) with a confidence level of 5% to determine the effect of each treatment on various observed variables.

RESULT AND DISCUSSION

Characteristics of Land and the Biogas Waste

The preliminary analysis is carried out on the land and biogas waste that will be used. Soil and waste were analyzed at the Laboratory of Soil Fertility, Faculty of Agriculture, University of Jember and also at the Jember Coffee and Cocoa Research Center. Analysis of organic C, ph, N, and P is available at the Laboratory of soil fertility while K determination is available at the coffee and cocoa research center of Jember district. Analysis of soil and biogas waste aims to determine the characteristics of soil and biogas waste that will be used for research experiments. The following are the results of the preliminary analysis of land and waste used can be seen in Table 1 and Table 2.

Based on the results of the analysis, nitrogen, phosphorus, and potassium are available in soil and waste. The results of the analysis are used to determine the treatment to be applied. Nutrient requirements for eggplant were 7.22g N, 3.36g P_2O_5 and 2.71g K_2O. Nutrients requitments for eggplant after be demished by nutrients on the 10 kg soil were 6.22 g N, 3.60 g P_2O_5 and 2.51

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K_2O. The following is a summary of the calculation of waste requirements as a substitute for nutrient requirements for eggplant.

Table 1. Characteristics of Early Soils before Research

Items of Analysis	Value	Unit	Standard	Method
C-Organic	%	1.49	Low	W & B
pH H_2O	-	6.76	Neutral	pH Meter
N	%	0.1	Very Low	Kjeldahl
P_2O_5	Ppm	14.12	Moderate	Olsen
K_2O	%	0.10202	Very Low	HCl 25%
C/N Ratio		14.9	Moderate	

* Criteria for Assessing the Results of Soil Analysis of the 2009 Soil Research Center

Source: Research Center of Kopi and Kakao Jember and Laboratory of Soil Fertility, University of Jember (2018)

The dose of waste used is based on nutrient requirements needed by eggplant, the number of nutrients in the soil and in the waste of biogas. Calculation of biogas waste requirements can be seen in Table 4. Adding different organic materials to the soil will affect the nature of the soil. Based on the results of the analysis after treatment, the following data are obtained.

Table 2. Results of Biogas Waste Analysis

Items of Analysis	Unit	Value	Quality Standart	Description
C-Organic	%	14.47	>12%	M
pH H_2O	-	7.15	4-8	M
N	%	0.77	2%	NF
P_2O_5	%	0.54	2%	NF
K_2O	%	0.25	2%	NF
C/N Ratio		18.79	10-25	M

* Regulation of Indonesia Agriculture Departement No. 28/Permentan/SR.130/B/ 2009.

Description: M = Meets, NF = Not Fulfilling

Source: Research Center of Kopi and Kakao Jember and Laboratory of Soil Fertility, University of Jember (2018)

Table 3. Nutrient Requirements for Eggplant

Required Nutrient	Kg Ha^{-1}	Gram $plant^{-1}$	Total plants Ha^{-1}	Required-soil
N (Urea)	200	7.22	27692	6.22
P_2O_5 (TSP)	100	3.61		3.60
K_2O (KCl)	75	2.71		2.51

Table 4. Biogas Waste Needs as a Substitute for Nutrient of Eggplant

Nutrion Subtitution	Required waste	Biogas Waste Wombs			Inorganic			Required Fertilizer		
	(Kg)	N	P_2O_5	K_2O	N	P_2O_5	K_2O	Urea	SP-36	KCL
0%	0.00*	0.00	0.00	0.00	2.32	1.65	1.42	3.87	4.57	2.37
20% N	0.60*	0.46	0.33	0.15	1.86	1.32	1.27	3.10	3.67	2.12
20 % P_2O_5	0.61	0.47	0.33	0.15	1.85	1.32	1.27	3.09	3.66	2.12
20%K_2O	1.14	0.88	0.61	0.28	1.45	1.03	1.14	2.41	2.87	1.90
40% N	1.21*	0.93	0.65	0.30	1.39	1.00	1.12	2.32	2.77	1.87
40 % P_2O_5	1.22	0.94	0.66	0.30	1.38	0.99	1.12	2.30	2.74	1.86
40%K_2O	2.28*	1.75	1.23	0.57	0.57	0.42	0.85	0.95	1.16	1.42

* Dose of biogas waste treated as treatment

Based on the results of the analysis there are differences in organic carbon content and cation exchange capacity (CEC) in the soil given biogas waste treatment. The land used for Eggplant planting media contains C-Organic as much as 1.49% which is included in the criteria of low C-Organic content. The highest C-Organic content is in the treatment of giving 2.2 kg $polybag^{-1}$ waste. The addition of sludge organic matter can increase C-Organic in the soil, which is 3.01% which is categorized as high (Table 5). The lowest organic C value is found in the treatment of 0 kg of waste $polybag^{-1}$ with a value of 1.53% organic C which is categorized as low.

The treatment of giving organic fertilizer to the soil with low organic C value can increase the C-organic content in the soil so that it can add nutrients. This addition of nutrients can influence on the growth and the yield of eggplant [8]. Based on the results of the research, increasing the dosage of organic fertilizer given increased the soil C-organic content.

Table 5. Characteristics of Soil after Waste Application

Treatment	Analysis	Value	Unit	Quality Standard*
0 kg	C-Organik	1.53	%	Low
	KTK	13.35	Cmol kg^{-1}	Low
0.6 kg	C-Organik	2.39	%	Moderate
	KTK	16.40	Cmol kg^{-1}	Low
1.2 kg	C-Organik	2.95	%	Moderate
	KTK	20.99	Cmol kg^{-1}	Moderate
2.2 kg	C-Organik	3.02	%	Moderate
	KTK	13.75	Cmol kg^{-1}	Low

* Criteria for Assessing Soil Analysis Result of the 2009 Soil Research Center

Source: Research Center of Kopi and Kakao Jember and Laboratory of Soil Fertility, University of Jember (2018)

Based on the results of CEC measurements after treatment, it was found that the highest CEC was found in the treatment of 1.2 kg of waste $polybag^{-1}$ with a value of 20.99 cmol kg^{-1} in

the medium category and the lowest CEC in the treatment of 0 kg waste polybag^{-1} with a value of 13.35 cmol kg^{-1} which was categorized low (table 5). But in general, the addition of sludge increases the soil CEC from before administration and after administration of sludge. This is because the sludge is included inorganic material so that it can increase CEC.

The increase in soil CEC due to the administration of organic matter is caused by decomposition of organic matter to produce organic compounds which can increase soil CEC [9]. The presence of organic matter in the soil chemical properties greatly affects the soil CEC in addition to the various nutrients contained in it which can also contribute to soil fertility [10].

CEC varies greatly on each type of land. The amount of soil CEC is influenced by the nature and characteristics of the soil itself, among others, soil reactions (pH), soil texture or amount of clay, types of clay minerals, soil organic matter, calcification, and fertilization [11]. Based on the results of the study, although the content of C-organic soil increases the CEC value does not necessarily increase because the CEC value is also influenced by several other factors [12]. This is possible in the treatment of 2.2 kg of the waste polybag^{-1} which is only KT 13.75 cmol kg^{-1}.

Effect of Biogas Waste on Growth and Yield of Eggplant

The addition of biogas waste from cow manure on planting eggplant is done to increase the availability and uptake of nutrients. The results of the diversity of the observed variables are shown in Table 6. Based on the results of variance analysis in table 6, it shows that there is a treatment effect on plant height variables, flowering age, harvesting age, fruit diameter and fruit weight of cropping.

Table 6. Summary of F count values and diversity coefficients on several observation variables

Observation Variabel	F-Count of dosage Treatments for Biogas	Coefficient of Varians
Plant Height	209.24**	16.18
Flowering Age	21.42**	19.92
Harvesting Age	69.16**	7.14
Fruit Diameter	22.40**	13.02
Fruit Weight per plant	64.27**	4.60

Description: **) Different is very real. *) Significantly different. ns): Different is not real

Eggplant Plant Height (cm)

Based on the results of the variance analysis in Table 7, the treatment of the sludge dose has a very significant effect on plant height on days 20, 30, 40, 50, and 60 and shows a different effect that is not significant on day 10. This situation occurs because of the large possibility young plants do not have perfect roots, consequently, the roots cannot absorb nutrients optimally. Young plants will be able to absorb nutrients in small amounts, in line with the age of the plant, the speed of absorption of plant nutrients will increase if the age increases; according to its life cycle. Increasing the age of the plant, the need for nutrients increases and decomposes perfectly which can be fulfilled by the soil where it grows. Organic fertilizers given still need time to experience decomposition [13].

Table 7. Effects of Giving Biogas Waste on the Height of Eggplant

Treatment	Day-				
	20	30	40	50	60
0 kg	10.00^{ab}	24.88^{a}	50.55^{b}	76.53^{b}	86.58^{b}
0.6 kg	10.03^{ab}	28.22^{b}	51.20^{b}	79.85^{c}	92.77^{c}
1.2 kg	10.65^{b}	31.15^{c}	55.07^{c}	82.10^{d}	102.80^{d}
2.2 kg	9.55^{a}	23.28^{a}	48.35^{a}	74.25^{a}	81.67^{a}

Organic fertilizer has a slow nature of providing nutrients for plants because it requires time for the decomposition process (slow-release). Fertilization using 100% inorganic has resulted in low efficient N absorption. This is due to N in Urea is fast release and immediately available for the plant, so that it is easily removed through washing, evaporation, and nitrification before it could be utilized by the plant. Deficient N during the generative phase will inhibit the formation and enlargement of the eggplant, and it will lead to low production [14].

The best treatment on plant height variables in the treatment of 1.2 kg polybag^{-1} waste dose with a final height of 102.8 cm. While plant height is strongly influenced by the presence of N elements in the soil. In untreated soils, the administration of organic material in the form of sludge shows a lower height (Table 7) even though it has been fertilized using inorganic fertilizers. This can be caused by the not optimum absorption of fertilizers applied. The factor of N loss rate from urea fertilizer is between 60-80% in paddy fields and 40-60% on dry land; so that only 30-50% can be used by plants. This can be caused by the nutrient washing process by water (leaching) around the plant so that N becomes unavailable. Nutrient N is also easy to evaporate. N which losses through the evaporation process (volatility) may reach 70% [15].

Flowering Age

Figure 1 shows that the eggplant plant was given the treatment by adding organic material in the form of waste as much as 1.2 kg polybag^{-1} has the average age of the fastest flowering and significantly different from the treatment without the provision of organic ingredients. The longest average age of flowering is found in the treatment of 2.2 kg of the waste polybag^{-1} with an average age of 35.83 HST. This can be caused by differences in the availability of P elements in each treatment.

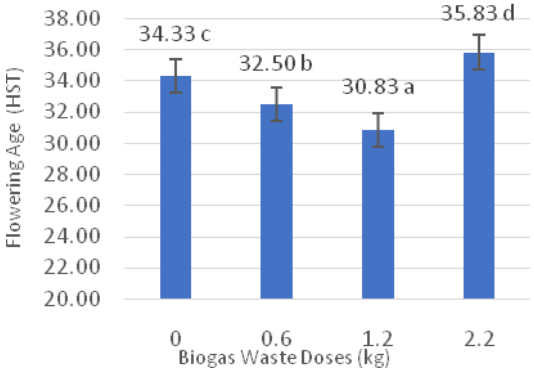

Figure 1. Effect of Giving Biogas Waste on Flowering Age of Eggplants

The availability of P elements for plants serves to accelerate the flowering and cooking of fruits, seeds or grain and accelerate the percentage of flower formation into fruit or seed. Planting media that have been given organic material has increased levels of P available, this is because phosphorus in the soil mostly comes from weathering of natural mineral rocks and the rest comes from weathering of organic matter. The form of phosphorus ions present in the soil also depends on the pH of the soil [16].

In the soil without the administration of organic ion P material, it will be bound by Al which is in the soil. The addition of organic material is one of the efforts that can be used to overcome the problem of civilization in the soil. Organic material in the decomposition process will release organic acids that can bind Al and form complex compounds so that Al becomes insoluble. Giving organic material is one way to accelerate the process of soil amelioration [17].

Harvesting Age

Figure 2 shows that eggplants are given the treatment of the addition of organic ingredients in the form of biogas waste 1.2 kg polybag^{-1} (L2) with the fastest average harvesting age (56.73 HST) and significantly different from the treatment without administration of organic material (L0) has an average harvest age of 60.17 HST. The longest average value of harvesting age was 2.2 kg of waste polybag^{-1} (L3) with an average of harvesting age of 61.03 HST. This can be caused by different elements in each treatment.

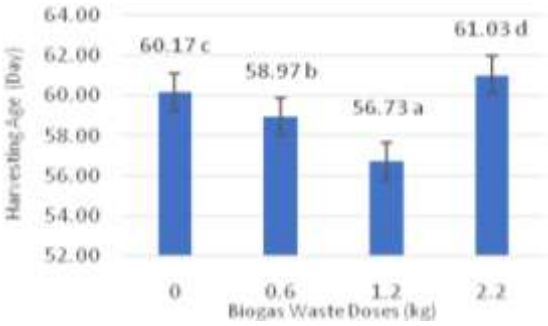

Figure 2. Effects of Giving Biogas Waste on Harvesting Age of Eggplants

The process of fertilizing and riping fruit requires sufficient N, P and K nutrients. In the soil treated with 1.2 kg of waste polybag^{-1} (L2), the highest CEC value was 20.99. The availability of nutrients in the soil can be seen from the ability of the soil to exchange cations (CEC), the higher the CEC, the higher the ability to supply nutrients to plants. The fermentation process in the digester occurs anaerobic overhaul of organic matter into biogas and acid which has a low molecular weight which causes the concentration of N, P and K to increase so that the CEC value of the soil that is given biogas waste is increased [18].

Fruit Diameter

Figure 3 showed that the best treatment to produce the largest diameter is the treatment of biogas waste 1.2 kg polybag^{-1} (L2) dose, with an average fruit diameter of 35.94 mm and the lowest treatment 2.2 kg polybag^{-1} (L3) with a flat fruit diameter 32.57. This can be affected by the availability of nutrients. Fruit shaping is strongly influenced by the availability of nutrients for photosynthesis which produce carbohydrates, fats, proteins, minerals and vitamins which will be translocated into storage [19].

In the treatment of biogas waste, 1.2 kg polybag^{-1} (L2) dose is able to provide a number of nutrients for generative development of plants, especially in the formation and ripening of fruit while in the treatment without giving waste (L0) and treatment of waste dose 2.2 kg polybag^{-1} (L3) fewer nutrients received. This can be seen in the CEC (Table 5) L2 land has a greater value than L0

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and L3. CEC values are related to the ability of the soil to provide nutrients to plants. The greater the value, the greater the ability of the soil to exchange its cations into plants.

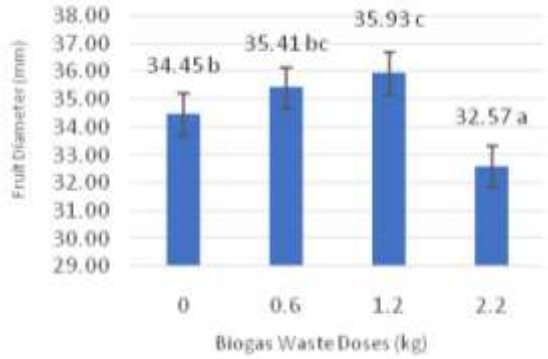

Figure 3. Effect of Giving Biogas Waste on Eggplant Diameter

Potassium has an important role in fruit shape. Potassium serves to increase the synthesis and translocation of carbohydrates for fruit filling [20]. Giving organic material that has an elemental content of potassium in the soil will increase the element of potassium, so potassium available for the soil will increase. The amount or amount of potassium absorbed by plants is strongly influenced by CEC (cation exchange capacity) and in general, soils with high CEC have the ability to store and provide greater K so vice versa, if the soil has a low CEC then the ability to store and provide K is also low [21].

Fruit Weight per Plant

Figure 4 shows that the best treatment to produce the best fruit weight per plant is the treatment of 1.2 kg polybag^{-1} biogas waste doses, having an average fruit weight of 1.36 kg plant^{-1}. Then, 2.2 kg polybag^{-1} is the lowest dose of biogas waste with an average value of 0.99kg plant^{-1} weight. This situation is caused by the provision of biogas waste from cow manure can meet the nutrient needs of both macro and micro plants for eggplants and also due to improvements in physical trait and soil biological properties, so that eggplants can thrive and produce high fruit production. Exchangeable nutrients can be seen through CEC values (Table 5).

Biogas waste manure contains macro and micronutrients and microorganisms that are still active in the process of ferments and decomposition. The more organic matter given to plants will show a good response to support the growth and production of more optimal eggplant fruit. Availability of nutrients is very important for plant growth and development because nutrient content will help facilitate the metabolic processes of plants, including photosynthesis, so photosynthates are produced high, then translocated throughout parts of plants, especially the storage of food reserves such as fruit [22]. The availability of these nutrients at appropriate levels is advantageous for the full development of the plants as they are uptaken by plants from the soil after application[23].

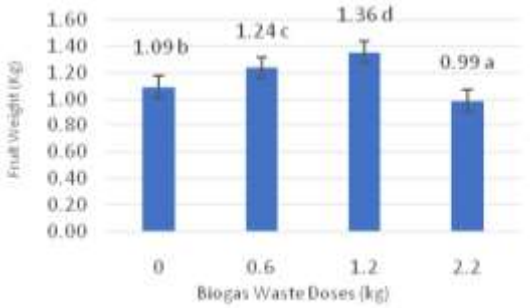

Figure 4. Effect of Biogas Waste on Fruit Weight Per Plant (Kg)

The application of biogas waste has not only provided nutrients, but also improving the physical, chemical, and biological features of the soil so that it could increase efficient absorption of the nutrients. Improvement on the soil features and the available nutrients have produced higher net assimilation rate for the eggplant [24]. Based on the research, the dosage of cow manure of biogas waste as an organic fertilizer affect the fruit weight of plants and crop production. the harvest yields of Leaf mustard were 2.3 and 2.2 times higher in the treatment supplied with inorganic fertilizers from co-digester [25].

CONCLUSION

Based on the research that has been done, it can be concluded that the best treatment for achieving optimum plant height, fruiting age, harvesting age, fruit diameter, fruit length, and fruit weight is the treatment of using biogas waste with the dose of 1.2 kg polybag^{-1} (L2), with a height of 102.8 cm, age of 30.83 HST, harvest age 56.73 HST, fruit diameter 35.93 mm, fruit length 17.95 cm and fruit weight of crop reached 1.36 kg (37.61 tons ha^{-1}).

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Optimization of Time and Temperature Gelatin Extraction from Pink Perch *(Nemipterus bathybius)* Head using Response Surface Methodology (RSM)

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Abstract

Gelatin from fisheries byproducts was very potential to be used as an alternative gelatin substitute for bovine and porcine gelatin. In this study, optimization of pink perch head gelatin extraction was carried out based on temperature and extraction time. Factorial design and Response Surface Methodology (RSM) were used to optimize the gelatin extraction process of pink perch (*Nemipterus bathybius*) head. The purpose of this research was to determine the optimum condition of the pink perch head extraction process based on the temperature and time of extraction. The extraction temperature (50˚C-90˚C), and the extraction time (3-7 hours) were the independent variables. The response variables of this study were the yield of gelatin (%), gel strength (g.bloom) and viscosity (cP). The optimum extraction conditions were obtained at the extraction temperature of 74.40˚C for 5.42 hours with a yield of 5.31%, the gel strength of 311.01 g.bloom and the viscosity of 5 cP. Considering its similarity to the commercial gelatin, particularly on their chemical characteristics, it can be suggested that the head of pink perch is a potential alternative of gelatin source.

Keywords: Extraction, Gelatin, Optimization, Pink Perch Head

INTRODUCTION

Gelatin is a polypeptide obtained from collagen degradation of animal's bone and skin which functions as an emulsifier, foam-former, gel-former and edible-film [1,2]. Gelatin is generally derived from bone and skin of bovine or porcine. However, the issue of BSE (Bovine Spongiform Encephalopathy) disease and differences in consumer belief is a problem for consuming bovine or porcine gelatin. Therefore, gelatin obtained from the industrial waste of fish processing can be used as an alternative source of gelatin [3].

The fish head is a waste produced by fish in the processing industry. Most of the waste is disposed and used to feed livestock [4]. Considering the chemical components of the fish head, its utilization as a source of gelatin production can effectively increase the value of waste economically and reduce environmental pollution as well [5].

To date, fish gelatin tends to be sub-quality compared to both bovine and porcine gelatin [6]. One of the factors that can affect the physicochemical properties of gelatin is the temperature and time of gelatin extraction. Several studies show that higher temperature and longer time of extraction can increase yield but decrease gel's strength and viscosity [7,8]. Therefore, the extraction condition of gelatin production needs optimization. Gelatin extraction with a variation of temperature and time has been reported for clown featherback [9], African catfish [10], seabass [2], wami tilapia [7] and channel catfish [11]. To date, there is few published works reported for gelatin extraction of pink perch head originated from Indonesia. The purpose of this research is to optimize temperature and extraction time of gelatin from pink perch *(Nemipterus bathybius)* head. Response Surface Methodology (RSM) was to establish an optimum condition of yield, gel strength and viscosity of gelatin. The optimum product then compared with commercial gelatin.

MATERIAL AND METHOD

Materials

The head of the pink perch (*Nemipterus bathybius*) was obtained from fisherman in the sea of Mimbo, Situbondo, East Java, Indonesia. The fish head was stored at -25°C until it was used. The chemical used are HCl (37%) Pro Analysis (PA).

Preparation of Raw Materials

Pink perch head separated from the body using a knife. Head washed with flowing tap water to remove dirt on the fish head and drain and then degreased by tumbling it in warm water

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at the temperature of 80°C for 20 minutes. Meat separated from the head, and then the pink perch head crushed using a knife to enlarge surface contact area.

Pre-treatment

Frozen pink perch head was thawed and drained. It was then weighed 100 g using analytical balance (Denver Instrument M-310) and soaked for 48 hours into 2.98% HCl (1:3 w/v) obtained from the previous study. The mixture was filtered using filter cloth. The collected residue (ossein) was washed by aquadest until reaching neutral pH and then it was ready for gelatin extraction.

Gelatin Extraction

The ossein obtained from the pre-treatment process was weighed using analytical balance (Denver Instrument M-310) and diluted into distilled water (1:3 w/v) and covered with aluminum foil. Sample was extracted using water bath with variation of extraction temperature (41.7, 50, 70, 90, 98.3°C) and extraction time (2.17, 3, 5, 7, 8.22 hours). The filtrate was filtered using filter cloth and dried inside a cabinet dryer at 50–55°C for 48 hours.

Table 1. Experimental Design of Gelatin Extraction

Independent Variable	Range and Level				
	-1.41	-1	0	1	1.41
Extraction temperature (°C) (X_1)	41.7	50	70	90	98.3
Extraction time (hour) (X_2)	2.17	3	5	7	8.22

Experimental Design

Central composite design (CCD) response surface methodology (RSM) was applied to analyze the relationship between independent variables and dependent variables on the gelatin extraction of pink perch head. The independent variables were extraction temperature (X_1, °C) and extraction time (X_2, hour), while the dependent variable was yield (Y_1, %), gel strength (Y_2, g.bloom) and viscosity (Y_3, cP). The experimental design is shown in Table 2. Statistically, the results of the central composite design experiments were analyzed with Design Expert 7.1.5. The purpose of optimization of gelatin extraction is to explain the effect of extraction temperature and time on yield, gel strength, and viscosity of gelatin from Pink perch head. Table 2 shows the result of the experiment from 2 factors with 5 levels ofcentral composite design, as the model of response surface method resulted from each response variable is shown on Table 3.

Yield of Gelatin

The yield was calculated by the ratio of the dry weight of produced gelatin and raw material used in the extraction process. The formula for yield as follows.

$$Yield = \frac{dry\ weight\ of\ gelatin}{weight\ of\ raw\ material}\ x\ 100\% \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Determination of Gel Strength

Gel strength measurement was prepared based on Ratnasari method [13]. Gelatin was dissolved in 60°C distilled water to a concentration of 6.67% (w/v). The mixture was stirred using magnetic stirrer (type MS200) until the gelatin was completely dissolved. The solution was incubated at 4°C for 18 h before analysis. Gel strength was measured by Tensile Strength Instrument (Imada/ZP-200N) Digital Imaging Model, load cell used 5 kg and 1 mm diameter Teflon Plunger Cylinder. The speed of the plunger was 0.5 $mm.s^{-1}$. Maximum strength (in grams) was taken at a penetration distance of 4 mm.

Table 2. The Experimental Design and Results for Gelatin Yield, Gel Strength, and Viscosity

Treatment	Code		Actual		Responses		
	X_1	X_2	Temperature(°C)	Time(hour)	Yield(%)	Gel Strength(g.bloom)	Viscosity(cP)
1	1.00	-1.00	50	3	1.01	57.78	3.33
2	1.00	-1.00	90	3	6.66	88.37	3.00
3	-1.00	1.00	50	7	2.39	163.15	3.67
4	1.00	1.00	90	7	8.10	47.59	2.67
5	-1.414	0.00	41.70	5	1.04	2.67	3.40
6	1.414	0.00	98.30	5	8.85	0.00	2.33
7	0.00	-1.414	70	2.17	1.95	149.56	3.33
8	0.00	1.414	70	7.83	5.45	214.14	3.67
9	0.00	0.00	70	5	4.52	309.31	5.33
10	0.00	0.00	70	5	5.04	302.51	4.67
11	0.00	0.00	70	5	4.90	319.51	5.00
12	0.00	0.00	70	5	4.81	316.11	5.67
13	0.00	0.00	70	5	4.50	305.91	4.67

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Table 3. Analysis of Variance (ANOVA) for the Response Surface Model of Yield, Gel Strength and Viscosity

Responses	Source	Sum of Squares	df	Mean Square	F-Value	p-value Prob > F
Yield	Model	72.17	5	14.43	113.09	< 0.0001
	X_1	62.75	1	62.75	491.66	< 0.0001
	X_2	7.54	1	7.54	59.11	0.0001
	X_1X_2	9.030E-004	1	9.030E-004	7.075E-003	0.9353
	X_1^2	0.16	1	0.16	1.24	0.3028
	X_2^2	1.55	1	1.55	12.16	0.0102
	Residual	0.89	7	0.13		
	Lack of Fit	0.67	3	0.22	3.89	0.1112
	Pure Error	0.23	4	0.057		
	Total	73.06	12			
Gel Strength	Model	1.912E+005	5	38245.53	238.29	< 0.0001
	X_1	984.48	1	984.48	6.13	0.0424
	X_2	3038.88	1	3038.88	18.93	0.0033
	X_1X_2	5339.96	1	5339.96	33.27	0.0007
	X_1^2	1.677E+005	1	1.677E+005	1044.80	< 0.0001
	X_2^2	29393.57	1	29393.57	183.14	< 0.0001
	Residual	1123.50	7	160.50		
	Lack of Fit	924.67	3	308.22	6.20	0.0551
	Pure Error	198.83	4	49.71		
	Total	1.924E+005	12			
Viscosity	Model	12.58	5	2.52	22.44	< 0.0001
	X_1	1.01	1	1.01	9.01	0.0199
	X_2	0.030	1	0.030	0.27	0.6203
	X_1X_2	0.11	1	0.11	1.00	0.3504
	X_1^2	8.50	1	8.50	75.79	< 0.0001
	X_2^2	4.32	1	4.32	38.50	0.0004
	Residual	0.78	7	0.11		
	Lack of Fit	0.032	3	0.011	0.057	0.9796
	Pure Error	0.75	4	0.19		
	Total	13.37	12			

Determination of Viscosity

Viscosity was determined following the method done by Ratnasari [13]. Gelatin was dissolved in distilled water (6.67% w/v). The solution was heated in a water bath at 60°C for 30 minutes. 20 ml of the gelatin solution was tested using Brookfield LVDV-II viscometer (Brookfield Engineering Laboratories Ltd., Middleboro, MA) with a small sample adapter and equipped with the No.1 spindle at 90 rpm.

Fourier Transform Infra Red (FTIR)

Fourier Transform Infrared spectra were determined using FTIR Spectrophotometer 8400S/Shimadzu within range of wavelength 400 - 4000 cm^{-1}.

RESULT AND DISCUSSION

Yield

The yield of gelatin from pink perch head was obtained between 1.01% until 8.85%. Analysis of variance (ANOVA) on yield suggests that the response following quadratic model as shown by R^2 = 0.9878. The result of Analysis of Variance (ANOVA) (Table 3) shows that variables X_1, X_2, and X_2^2 have a significant effect on the response of gelatin yield with P <0.0001 and 0.0102. There is no interaction between treatment of extraction time and temperature (P = 0.9353). The equation below is the response of gelatin's yield with quadratic model:

$$\text{Yield} = 4.75 + 2.80X_1 + 0.97X_2 + 0.015X_1X_2 + 0.15X_1^2 - 0.47X_2^2 \quad (2)$$

The equation showed that the yield of gelatin increased alongside the increase of extraction temperature and time extraction respectively (Fig. 1). This result also reported on the previous research by using clown featherback [9], browbanded bamboo shark and blacktip shark [14], seabass [2], Atlantic cod, salmon and Atlantic herring [15].

Higher temperature increases kinetic energy required to hydrolyze triple helix bond of collagen which is water insoluble into water-soluble gelatin with α and β chain. Lengthening extraction time may increase the energy supply to hydrolyze collagen [2,15].

Gel Strength

The gel strength of gelatin extracted from Pink perch head was 0.00 g.bloom (gel isn't formed) until 319.51 g.bloom. The chosen model

in the analysis of variance was a quadratic model because of its significance on the response of gelatin's gel strength, moreover, it had highest value of R^2 (R^2 = 0.9942) and insignificant lack of fit (P = 0.0551).

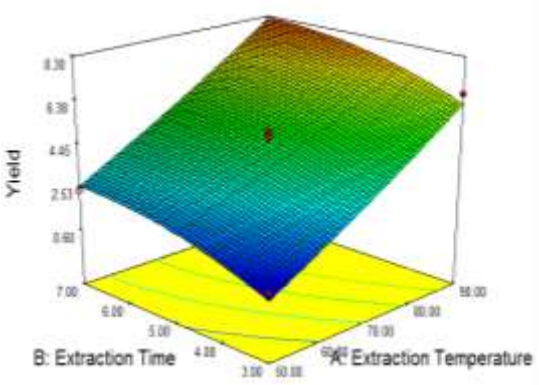

Figure 1. Surface Profile of Yield

The results of analysis of variance (ANOVA) on gel strength with quadratic model shows that variables X_1, X_2, X_1X_2 significantly affect gelatin's gel strength with P-value were 0.0424, 0.0033 and 0.0007. The value of X_1^2 and X_2^2 also have significant effects with P <0.0001 (Table 3). The equation of gelatin's gel strength with quadratic model is:

$$\text{Gel strength} = 310.67 - 11.09X_1 + 19.49X_1X_2 - 36.54X_2 - 155.26\,X_1^2 - 65X_2^2 \quad \text{.....(3)}$$

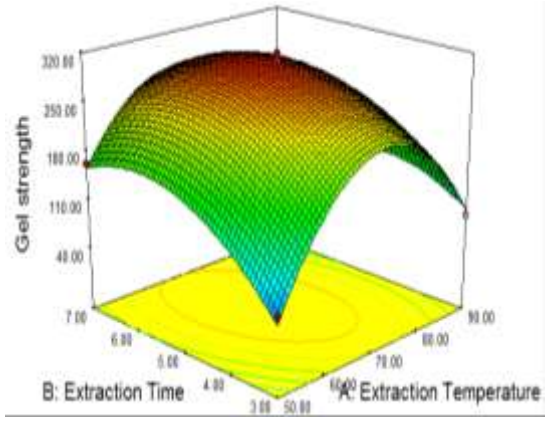

Figure 2. Surface Profile of Gel Strength

The strength of gelatin's gel will decrease with the increase of temperature and time extraction. Low extraction temperature and time also will produce gelatin with low gel strength. High temperature and time of extraction will result in overly degraded protein, thus producing protein fragment with low gel strength properties. Gelatin's molecules with short-chain cannot form strong junction zone, especially hydrogen bonds or weaker bonds such as hydrophobic bond and ionic bond [9,16]. Chemically, the formation of gel begins with the formation of Junction Zone within the three-dimension network of gelatin chain. Gelatin with low molecular weight will disrupt renaturation of helix bond, renaturation is a recombining process of gelatin strains into helical structure upon cooling. Renaturation is a part of junction zone formation, high molecular weight gelatin with branched structure and non-linier chain can slow down the renaturation process. Gelatin with high molecular weight is suspected of being produced from partial hydrolysis due to a lower temperature and shorter time of extraction. Higher or lower temperature than the optimum condition will weaken the gel strength of gelatin. [17]. The graph of contour plot and surface response profile of gel strength is shown in Figure 2.

Viscosity

The viscosity of gelatin extracted from pink perch Head was around 2.67 cP until 4.67 cP. Analysis of Variance (ANOVA) of viscosity used quadratic model because of high value of R^2 = 0.8993 and significant model (P < 0.0001) with insignificant value of lack of fit (P-value= 0.9796). The result of Analysis of Variance (ANOVA) on the response of gelatin's viscosity shows that quadratically variables X_1, X_1^2, X_2^2 give significant effect on viscosity with each P-values as following 0.0199, <0.0001 and 0.0004. Variable X_2 and X_1X_2 have no significant effect on theviscosity of gelatin with the P-value = 0.6203 and 0.3504 (Table 3). The equation of the response of gelatin's viscosity with quadratic model is:

$$\text{Viscosity} = 5.07 - 0.36X_1 + 0.0617X_2 + 0.17X_1X_2 - 1.11X_1^2 - 0.79X_2^2 \quad \text{.....(4)}$$

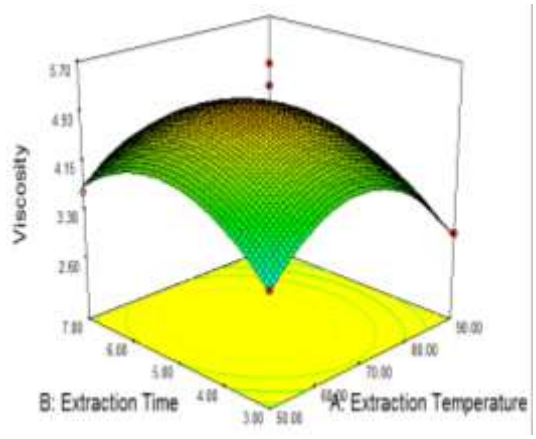

Figure 3. Surface Profile of Viscosity

The viscosity of the gelatin tends to increase when the temperature and time of extraction increase, but the viscosity will start to decrease back at certain high extraction temperature and time. It has been reported that viscosity of Pink perch's skin and bone are 8.47 cP and, 6.8 cP [18]. Viscosity can be affected by molecular weight and hydrodynamic interaction of intermolecular of open-chain polypeptide [19,20]. A low number of the β chain in the gelatin molecule caused low viscosity of gelatin from tilapia fish [21]. The graph of contour plot and surface response profile of gelatin's viscosity is shown in Figure 3.

Multiple Response Optimization

Optimization was done after a mathematic formula had been obtained for each response. The purpose of the optimization was to minimize the required effort and to maximize expected results. From this approach, the optimum condition was stabilized by desirability value. Determination of optimum point criteria is shown in Table 4.

After determining the criteria of optimum conditions, Design Expert program decided optimum solution with the highest value of desirability (approaching 1), only one optimum solution came out which is shown in Table 5. The next step was the verification of the results of the optimum solution's prediction (Table 5).

The optimum values of the gel strength and viscosity were compared to those of commercial gelatin. Gel strength of gelatin from pink perch was 311.01 g.bloom, while the gel strength of commercial gelatin was 364.54 g.bloom. Based on the result of independent t-test, the gel strength of gelatin from pink perch and commercial gelatin are significantly different with P-value = 0.023 (P< 0.05). Statistically, there is no significant difference in viscosity of the pink perch gelatin and commercial gelatin with P-value = 0.059 (P> 0.05).

Fourier Transform Infrared (FTIR)

The results of functional group characterization using FTIR *(Fourier Transform Infra Red)* showed specific vibration which was emitted from each functional groups within a certain wavelength. Gelatin from pink perch had similar spectra of FTIR to that of commercial gelatin (e.g Fig.4 and Table 6). Amida A band of gelatin from pink perch was found in the frequency of 3300 cm^{-1} and commercial gelatin was in the frequency of 3293 cm^{-1}. Amida A band shows there is a stretching vibration of theN-H group. Frequency of free N-H group is around 3400 – 3440cm^{-1} [14]. The frequency will decrease when the N-H group is involved with the formation of ahydrogen bond from α-chain gelatin with a range of frequency 3289 – 3304 cm^{-1} [2,23].

Amida B band of gelatin from pink perch and commercial gelatin were detected in the frequency of 3080 cm^{-1} and 3074 cm^{-1}. Amida B band shows stretching vibration of an asymmetric group of =C-H and NH_3^+. Some of the previous research mentioned that Amida B band was in the frequency of 3071 – 3079 cm^{-1} [2] and 3080 – 3087 cm^{-1}, the low frequency of Amida B band was suspected as a result of interaction between NH_3^+ group with peptide chain [24]. Therefore, the low frequency of the Amida B band from pink perch gelatin is due to the interaction between NH_3^+ group with the peptide chain.

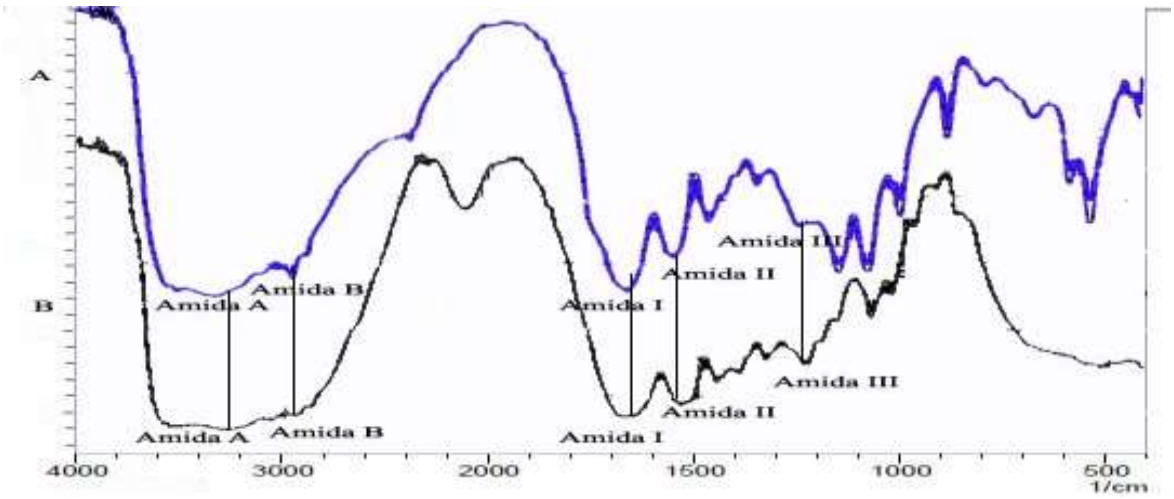

Figure 4. FTIR. A) FTIR spectra of commercial gelatin; B) FTIR spectra of pink perch gelatin

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Table 4. Criteria of Optimum Condition

Criteria	Goal	Limit Values	
		Low	High
Extraction Temperature (°C)	In range	41.7	98.3
Extraction Time (hours)	In range	2.17	7.83
Yield of Gelatin (%)	Maximize	1.01	8.85
Gel Strength of Gelatin (g.bloom)	Maximize	0.00	319.51
Viscosity of Gelatin (cP)	Maximize	2.33	5.67

Table 5. Optimal condition for Gelatin Extraction from Pink Perch Head

Condition for Gelatin Extraction	Independent Variable		Responses			Desirability
	Extraction Temperature (°C)	Extraction Time (hours)	Yield (%)	Gel Strength (g.bloom)	Viscosity (cP)	
Optimum Solution	74.40	5.42	5.56	300.21	4.91	0.749
Verification	74.40	5.42	5.31	311.01	5.00	
Precision Level			95.50%	97.48%	98.17%	

Table 6. Functional Group of FTIR Spectra

NO	Functional Group	Frequency		
		Commercial Gelatin	Pink Perch Gelatin	References*
1	Amida A (N-H Stretching vibration)	3293 cm^{-1}	3300 cm^{-1}	3289-3304 cm^{-1}
2	Amida B (=C-H and NH_3^+ Asymmetric Stretching vibration)	3074 cm^{-1}	3080 cm^{-1}	3071-3087 cm^{-1}
3	Amida I (C=O Stretching vibration)	1653 cm^{-1}	1649 cm^{-1}	1632-1653 cm^{-1}
4	Amida II (N-H Bending and C-N Stretching)	1541 cm^{-1}	1539 cm^{-1}	1540-1543 cm^{-1}
5	Amida III (C-N stretching and deformation of N-H group)	1238 cm^{-1}	1233 cm^{-1}	1233-1239 cm^{-1}

***Sources:** (1) Doyle and Chemistry, 1975 [23]; Sinthusamran, et al., 2014 [2]. (2) Sinthusamran, et al., 2014 [2]; Nagarajan, et al., 2012 [24]. (3) Nagarajan, et al., 2012 [24]. (4) Sinthusamran, et al., 2014 [2]. (5) Sinthusamran, et al., 2014 [2]; Kittiphattanabawon, et al., 2010 [14].

Amida I band of commercial gelatin was found in the frequency of 1653 cm^{-1}, as the one of gelatin from pink perch was in the frequency of 1649 cm^{-1}. Amida I band shows there is stretching vibration of C=O group in the secondary structure of the protein [14]. The range of frequency band length of Amida I is 1632 – 1635 cm^{-1} [24]. The frequency of Amida I can be affected by the molecular weight of gelatin. A large number of low molecular weight gelatin will make the C=O more exposed and more reactive thus the frequency of Amida I band will increase. This happens during high extraction time.

The frequency of Amida II band in commercial gelatin was 1541 cm^{-1} and the frequency of gelatin from pink perch was 1539 cm^{-1}. Amida II shows bending vibration of the N-H group and stretching vibration of C-N group. The range of frequency of Amida II band is 1540 – 1543 cm^{-1} [2]. Amida III band of commercial gelatin was 1238 cm^{-1}, and the frequency of gelatin from pink perch was 1233 cm^{-1}. The previous research mentioned that Amida III band was in the frequency of 1233 – 1234 cm^{-1} [2] and 1237 – 1239 cm^{-1} [14]. Amida III band shows a combination of C-N and N-H vibration stretching movements which are deformed, and it also shows the wagging vibration of CH_2 end-chain of glycine and side-chain of proline. Amida III band explains the degradation of triple helixstructure of collagen into gelatin with a much simpler structure [19,25,26].

Aside of Amida band frequency, the peak was found in commercial gelatin with quite high intensity in the frequency 1163 cm^{-1}, 1080 cm^{-1}, 1030 cm^{-1}, and 974 cm^{-1}, and the frequency of pink perch gelatin were 1138 cm^{-1}, 1067 cm^{-1}, and 988 cm^{-1}. The bands showed stretching vibration of C-O group in short peptide chain, and an indication of peptide chain degradation [25, 26].

CONCLUSION

This study showed that optimum extraction condition from pink perch head was obtained at 74.40°C for 5.42 hours. The results of characterization using FTIR *(Fourier Transform Infra Red)* found specific vibration from Amida A, Amida B, Amida I, II, and III.

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Effects of Monosodium Glutamate Oral Administration on LH and Testosterone Levels in Serum of Adult Male Rats (*Rattus norvegicus*)

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Abstract

This study aims to determine the effect of monosodium glutamate on LH and testosterone levels in serum of adult male rats (*Rattus norvegicus*). A total of 12 male Wistar-strained rats divided into three groups with 4 rats per group. Control group was given distilled water, while M1 was given MSG at a dose of 4mg.gBW^{-1} for 15 days and M2 was given MSG at a dose of 4 mg.gBW^{-1} for 45 days. All substances were given orally. At the end of the study, rats were sacrificed with a dislocation to draw blood from the heart. Then, the blood was incubated at room temperature to obtain serum. Serum was used to analyze LH and testosterone levels using the ELISA kit. Data were analyzed using one way ANOVA ($p \leq 0.05$) with SPPS 16.0 for windows. The results showed that serum LH levels after MSG treatment for 45 days was significantly decreased compare with the control. The serum testosterone levels M2 was significantly different from the control group ($p \leq 0.01$). This study predicts that monosodium glutamate have a direct and indirect effect on male reproductive organs.

Keywords: Luteinizing hormone, monosodium glutamate, testosterone.

INTRODUCTION

In the past few decades, monosodium glutamate (MSG) has become the most famous flavoring used throughout the world [1,2]. MSG can be easily found in packaged foods, fast food, and household kitchens. MSG has been categorized as a flavor enhancer that is safe for consumption. However, its use is increasing globally including in Asia with 4 g.day^{-1} and in Europe around 1 g.day^{-1} [3]. Progress in the practice of food production and the need for food preservation cause the advised amount of MSG consumption is exceeded in a short time. Consumption of MSG allowed by the FDA (Food and Drug Administration) and the WHO (World Health Organization) is 120 mg.kg^{-1} day [4].

Excessive consumption of MSG has a detrimental effect on humans and experimental animals [1,5]. MSG can cause damage to the male reproductive system by increasing Reactive Oxygen Species (ROS) in the brain [6] and testicles [7]. Excessive MSG exposure can increase L-glutamate level in the blood which then spreads throughout the body, especially in the brain and testicles. The high levels of L-glutamate cause overstimulation of the glutamate receptor resulting in increase intracellular Ca^{2+} ions [8]. This causes excessive entry of calcium ions into the mitochondria and consequently increases the production of ROS [9]. The excitotoxicity effect of MSG causes interference with the hypothalamic-pituitary-axis (HPA) pathway [10]. Disruption in the HPA pathway decreases the production of gonadotropin-releasing hormone (GnRH). It decreases luteinizing hormone (LH) released from the anterior pituitary. Reduced stimulation of LH in Leydig cells decreases testosterone production which ultimately leads to decreased sperm concentration [1,11]. Increased ROS in the testes caused lipid peroxidation resulting in disruption of membrane permeability and ATP synthesis which results in death in Leydig cells [12,13]. Decreasing the number of Leydig cells decreases the production of testosterone in the testis [14].

Sarhan [15] reported, MSG at a dose of 6 mg.gBW^{-1} per day for 45 days in male rats increased the production of reactive oxygen species (ROS) which was marked by an increase in MDA levels significantly. In addition, there is damage in the testis in the form of a decrease in the diameter and height of the epithelial layer in the seminiferous tubules. Spermatogenic cells, Sertoli cells, and Leydig cells show irregular nuclei, cytoplasmic vacuolation, and swelling of the mitochondria. Farombi and Onyema [6] mentioned intraperitoneal administration of a dose of 4 mg g^{-1} BW in male rats caused an increase in ROS in the brain. This results in damage to the male reproductive system [16].

Therefore, this study aims to explain the effect of MSG on LH and testosterone levels

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serum in male rats (*Rattus norvegicus*). Male rats were given oral MSG at a dose of 4 mg.gBW^{-1} (a third of LD50) for 15 days and 45 days.

MATERIALS AND METHODS

Research Materials

Total of 12 sexually mature male rats (*Rattus norvegicus*) with a weight of 200-300 g aged 3-4 months were used in this study. The experimental animals were kept in standard clean rat cages (32 x 28 cm) with *rodent pellets* and water available *ad libitum*. The animals were acclimatized for 2 weeks before and during the study at *Animal House,* the Laboratory of Structure Physiology and Animal Development, Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang.

Monosodium glutamate

In this study, the daily dose of MSG was 4 mg g^{-1} BW given orally to male rats. MSG was obtained from the local market in Malang.

Experimental design

Total of 12 rats chosen randomly were divided into 3 groups (n =4). The groups consisted of a control group (M0), MSG treatment group M1 and M2.

M0 (control group) : distilled water for 45 days
M1 (treatment MSG 1): 4 mg.gBW^{-1} for 15 days
M2 (treatment MSG 2): 4 mg.gBW^{-1} for 45 days

After given MSG, all experimental animals were dislocated and blood was drawn from the heart. The obtained blood was incubated at room temperature for 6 hours. After that, the serum obtained was stored at -20^{o}C.

Testosterone Serum and LH Assay

Testosterone and LH concentrations in serum were analyzed using Rat Testosterone ELISA kit 96T (E0259Ra) and Rat LH ELISA kit 96T (E0179Ra) the Bioassay Technology-Laboratory.

Statistical Analysis

Data were analyzed using one-way ANOVA ($p<0.05$) with SPSS 16.0 for windows.

RESULTS AND DISCUSSION

Effects of MSG on serum levels of LH

Figure 1(a) presents the decrease of LH levels along with the length of MSG exposure. However, in the statistical test, the MSG treatment group for 15 days (M1) did not show a significant difference with the control group. While the MSG treatment group for 45 days (M2) show a significant difference with the control.

In this study, the LH level of M2 was significantly different from the control (M0). This study conducted by Ochiogu *et al.* [1] which stated that MSG can significantly reduce LH level. MSG is reported to have an indirect effect on male reproductive organs through interference with the pathway of the HPA [17]. MSG can cause an increase in L-glutamate level in the hypothalamus resulting in intracellular calcium (Ca^{2+}) influx [18].

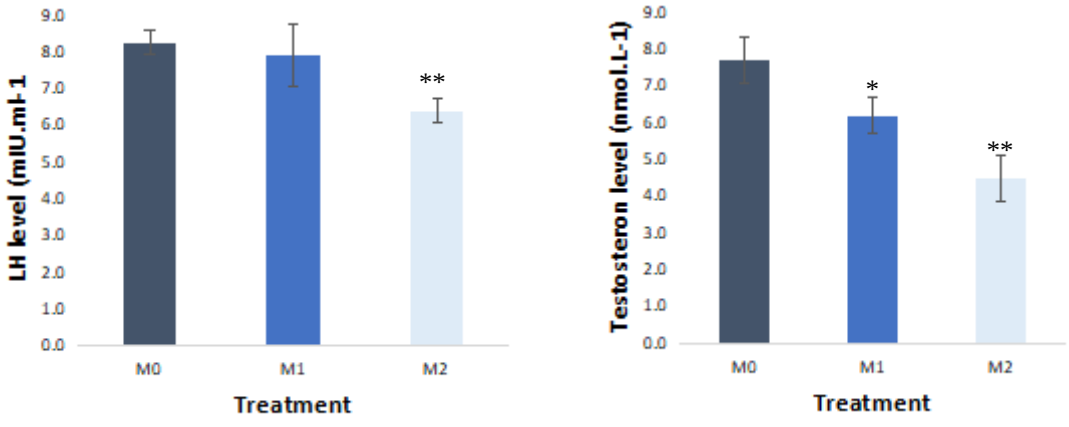

Figure 1. LH and Testosterone Level in Rats Serum

Description: (a) LH serum levels (mIU.ml^{-1}) determined after treatment in control (M0) and MSG-treated groups (M1: MSG for 15 days and M2: MSG for 45 days), (b) Testosterone serum levels (nmol.L^{-1}) determined after treatment in control and MSG-treated groups. Statistically significant difference from control showed by *p<0.05; **p<0.01.

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L-Glutamate can activate NMDA receptors in the hypothalamus causing large quantities of calcium ions (Ca^{2+}) along with sodium ions through the AMPA receptor. The excessive concentrations of calcium ions (Ca^{2+}) in the intracell results in mitochondrial dysfunction followed by the release of free radicals and the stimulation of the MAPK protein p38 (*mitogen-activated protein kinase p38*) which activates transcription factors. This results in damage and neuronal cell apoptosis [19,20]. Park *et al.* [21] reported, MSG at a dose of 4 mg g^{-1} BW in adult rats gave rise to neurodegeneration in the form of fewer neurons than controls. It was also found damage to the nucleus arkuata in the hypothalamus which played a role in GnRH regulation. The decrease in GnRH influences the release of LH from the anterior pituitary [1]. LH interacts with receptors on Leydig cell membranes to synthesize and secrete testosterone.

Effects of MSG on serum levels of testosterone

The average testosterone concentration of group M1 (MSG 4 mg.gBW^{-1} for 15 days) was significantly lower compared to the control group. M2 Group (MSG 4 mg.gBW^{-1} for 45 days) had a very significant difference with the control group ($p \leq 0.01$) (Fig. 1b).

Decreased testosterone can occur due to decreased LH level [1]. In this study, the decrease in LH did show a significant difference after MSG treatment for 45 days. In addition, a decrease in serum testosterone was probably caused by damage and a decrease in the number of Leydig cells in the testis. Leydig cells are cells that play a role in producing the testosterone hormone located between seminiferous tubules (interstitial space) [22]. According to a study by Suryadi [23], the Wistar strain of male rats (*Rattus norvegicus)* given a dose of 4800mg kg^{-1} BW/day MSG for 49 days showed that the Leydig cell's core diameter was smaller than those in control group. In addition, the number of Leydig cells decreased significantly compared to those in the control group. This decrease in the number of Leydig cells was caused by an increase in the production of ROS in testis [7].

Increased ROS is caused by a high level of L-glutamate due to excessive consumption of MSG for a long time [24,25]. L-glutamate in high concentrations in the testis can activate excess glutamate receptors. The activation of the glutamate receptor can lead to the increasing of Ca^{2+} intracell. Natrium enters and triggers cell depolarization which causes activation of NMDA receptors. Therefore, Ca^{2+} can enter intracell [26]. Ca^{2+} ions which enter into mitochondria in excessive amounts cause deviations in mitochondrial electron chain function leading to excessive production of ROS [27]. ROS is released in the form of O_2 and then converted to H_2O_2. Then, hydrogen peroxide (H_2O_2) reacts with Fe^{2+} through a Fenton reaction that forms OH^- and lipid peroxidation. Lipid peroxidation causes damage to cell membranes, especially the content of phospholipids, DNA damage, and protein molecules [9]. This can lead to ruptured cell membranes and necrosis, causing a decrease in the number of Leydig cells [12]. The decrease in the number of Leydig cells effects the testosterone production and testosterone levels in the testes [14]. In the study of Igwebuike *et al.* [28], oral administration of monosodium glutamate at a dose of 4 mg g^{-1} BW in Sprague-Dawley rats every 48 hours for 6 weeks can reduce sperm reserve in cauda epididymis ($P<0.05$) and serum testosterone levels significantly.

CONCLUSION

Based on the results of this study, it can be concluded that the administration of MSG in male rats (*Rattus norvegicus*) can cause a significant decrease in LH and testosterone. This study predicts that monosodium glutamate have a direct and indirect effect on male reproductive organs.

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Dietary Rice Bran Plays A Significant Role in the Hepatoprotective Effect in Hypercholesterolemic Rats

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Abstract

Cholesterol is obtained through biosynthesis and diet. When a level of cholesterol is above the normal level, this condition caused hypercholesterolemia. Long-term administration of synthetic chemical drugs can cause liver damage. Therefore, alternative natural medication is needed. One of the alternatives that can be used is the rice bran (RB), which contains antioxidant and crude fiber. This study is aimed at finding out the potential utilization of RB on total cholesterol level, liver enzyme as aspartate aminotransferase (AST) and alanine aminotransferase (ALT), malondialdehyde (MDA), and the changes in liver tissue. This study uses five groups of rats: the negative control group, positive control group, and three therapy groups with the dosage of 270 mg kg^{-1}, 540 mg kg^{-1}, and 810 mg kg^{-1} of body weight. This study shows that therapy using RB can significantly decrease the cholesterol level, AST, ALT, and MDA ($p<0.01$). The total cholesterol level is 21%, AST and ALT activities can be reduced to 54% and 64%, the level of MDA reduced to 79% and can repair the liver tissue. This study shows that RB can be effectively used as hepatoprotective in rats with hypercholesterolemia.

Keywords: AST and ALT, Hypercholesterolemia, Rice Bran, Total Cholesterol Level.

INTRODUCTION

Hypercholesterolemia is influenced by age, sex, alcohol, obesity, stress, diabetes, and food intake [1,2,3]. The excessive consumption of fatty food can increase the level of cholesterol in the blood. Hypercholesterolemia can become the risk for atherosclerosis, pancreatitis, coronary heart, thyroid disorder, kidney failure, and liver damage [4].

Hypercholesterolemia indicates the existence of free radical within the body due to the cholesterol metabolic disorder. The increase of free radical can stimulate the peroxidation of lipid, hence, cause oxidative stress which can be measured using the MDA parameter [5]. The increase of free radical can reduce the activity lipoprotein lipase enzyme (LPL). This can cause a disturbance in the changes of very low-density lipoprotein (VLDL) into intermediate density lipoprotein (IDL). Therefore, VLDL will form a sediment in the liver and cause fatty in the liver, such as infiltration of fat into the surrounding of hepatocyte cells and sinusoid [6].

The cells damage within the liver can be clinically known at advanced stage. However, ongoing liver damage can be known by measuring the parameter of liver function [7]. The indicator of the damage of liver cells is the increase of liver enzymes within the serum, such as AST and ALT enzymes [8].

Nowadays, several medicines have been reported to have cure Hypercholesterolemia which consists of statin, niacin, fibrate acid, nicotinate acid, and resin [9,10,11]. However, long-term medication of hypercholesterolemia using synthetic chemical medicine can cause liver damage and kidney functions disorder [12]. Thus, alternative medication from nature is needed. One of the alternatives for this medication of hypercholesterolemia is the utilization of RB.

RB has nutrition such as carbohydrate, protein, mineral, fat, and crude fiber [13,14,15]. In addition, RB also contains bioactive components such as phenolic compounds, oryzanol, vitamin E (Tocopherol and Tocotrienol) which serve as an antioxidant [15,16,17]. Several types of research have reported that rice bran can reduce the total cholesterol level, triglyceride, LDL, and increases the concentration of HDL [18,19]. In addition to finding out the influence of RB toward the

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cholesterol level, this study is also carried out to find the role of RB as hepatoprotective in rats with hypercholesterolemia, by using the liver function parameters of activity of the AST and ALT enzymes, MDA in liver organ, and supported by the observation of the liver tissue.

Moreover, RB also contains antioxidants that can prevent oxidative stress and inhibit the process of fat oxidation, and also contains crude fiber which can bind cholesterol [9]. This study will examine the effect of RB on total cholesterol levels, liver enzymes activity (AST and ALT), and MDA levels.

MATERIAL AND METHOD

Material and Animals

This study uses RB from white rice (*Oryza sativa*) from Anugrah Brand. The animal used in this study is 20 male Rats (*Rattus norvegicus*) age 2 months old, with the average weight of 200 gram. All the procedure for this animal usage has been approved acceptance of Brawijaya University's Research Commission number 941-KEP-UB.

Analysis of crude fiber content in rice bran

One gram of RB is inserted into 300 mL Erlenmeyer tube. Then, the RB is added with 0.3 N H_2SO_4 under the cooling back. The sample is boiled for 30 minutes and often shook. The sample is filtered using the filter paper. The residue is washed using the boiling water, hence, no longer acidic (tested using the Litmus paper). The residue then is inserted into the Erlenmeyer tube. The residue left in the paper then washed using 200 mL of boiling NaOH hence all the residue entered the Erlenmeyer tube. The sample is boiled for another 30 minutes. Then the sample is washed with 105 K_2SO_4 solutions. The residue is then washed using 15 mL of 95% alcohol, and then the filter paper is dried in the temperature of 110^0C hence, the weight is constant then the sample is weighed.

$$\text{The crude fiber level (\%)} = (A\text{-}B)/C \times 100\%$$

Description:
A : the weight of the filter paper+residue)
B : the weight of empty filter paper
C : sample weight

Determining the level of Antioxidant using the DPPH test

The antioxidant activity is determined by using the DPPH test which through the procedure adapted from Dudonne [20]. 1 mL of DPPH solution (0.05 mM) is mixed with 2.5 rice bran extract. Then the mixture is incubated in the temperature of 37^0C for 20 minutes. The absorbance is measured using spectrophotometer UV-Vis in the length of wave 515 nm. Then, 2.5 mL of methanol is mixed with 1 mL of DPPH solution, and this mixture is used as a blank solution. The percentage of antioxidant activity to prevent the free radical (IC_{50}) can be calculated using the formula:

$$\text{\% inhibition} = (\text{A Blank} - \text{A Sample})/\text{A Blank} \times 100$$

Induction of High Cholesterol Diet and Treatment

The rats is distributed into 5 groups, each group with 4 rats: (1) negative control group, (2) positive control group, (3) RB therapy dosage is 270 mg kg^{-1} bw, (4) RB therapy dosage is 540 mg kg^{-1} bw, and (5) RB therapy dosage is 850 mg kg^{-1} bw. Each mouse is fed with a high cholesterol meal, except for the negative control group. The food is administered for 8 weeks. The composition of high cholesterol meal consists of 32 g of goat fat, 0.32 g of folate acid, 16 g of cooked quail yolk eggs, and 4 mL of used cooking oil. The cholesterol level is tested every 14 days. The rats with a cholesterol level of more than 200 mg dL^{-1} will be treated with rice bran with the previously mentioned dosage. On the 15^{th} day, the rats will be operated to take the blood and liver organ samples. The serum is taken after it is being separated from blood.

Measurement of total cholesterol level in serum

Total cholesterol level is tested using the CHOD-PAP reagent cholesterol method. The total cholesterol level is measured by adding 10 µL serum with 1000 µL reagent. After incubated, the absorbance is read using the spectrophotometer UV-Vis in the length of wave 520 nm.

Measurement of Aspartate and Alanine Aminotransferase Activities (AST and ALT)

The AST and ALT level is tested using the IFFC method. The activity of AST enzyme is measured by adding 500 µL reagent AST in 50 µL serum. Whereas, the measurement of ALT enzyme is measured by adding 500 µL reagent ALT in 50 µL serum. After the mixture is homogeneous, each absorbance is read using the spectrophotometer UV-Vis in the length of wave 340 nm.

Measurement of Malondialdehyde (MDA)

Supernatant formed from the isolation of protein liver organ is taken by the amount of 100 µL. The supernatant then added with 550 µL distilled water and 100 µL trichloro ethanoic acid, then the sample is centrifuged. The result is added with 250 µL HCl 1 N and centrifuged. The

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supernatant then added with 100 µL of 1% Na-Thio and the result is made homogeneous. After that, the supernatant is heated in the temperature of 100^0c for 30 minutes. This supernatant the centrifuged in 500 rpm for 15 minutes. Following the incubation, the sample is read using the spectrophotometer UV-Vis in the length of wave 533 nm.

Histopathological Examination

The liver organ is taken from the operated mice then washed using the 0.9% of NaCl physiology. The preparation stage includes fixation, dehydration, cleaning, soaking, splitting, and attachment to the glass object. The rat liver is prepared for hematoxylin and eosin (HE) coloring. The rat liver is observed its histopathology description by using the BX51 Olympus microscope in the magnification of 100x and 400x.

Statistical Analysis

All the value is stated in the means ±SD. The data are analyzed using the ANOVA and post-hoc LSD test, and the highly significant result in $p<0.01$.

RESULT AND DISCUSSION

The influence of rice bran toward the total cholesterol of rats with hypercholesterolemia

Based on Tabel 1 below, the RB in the dosage of 270 mg kg^{-1} bw, 540 mg kg^{-1} bw, and 850 mg kg^{-1} bw can significantly decrease the total cholesterol level in mice with hypercholesterolemia with the percentage of 7%, 17%, and 21% respectively compared to the positive control group. According to several previous studies, RB can inhibit the increase of cholesterol, and decrease the cholesterol level and lipid profile.

RB has the characteristic of reducing cholesterol called cholesterolemic effect. RB has 10.89% crude fiber content. This fiber is able to absorb lipid from the digestive channel. This fiber is also able to bind the bile acid and cholesterol that can be disposed with feces. Therefore, the radical bile acid will decrease and new bile acid will be needed. The more the new bile acid produced from the absorption of cholesterol in the blood, the more cholesterol will be excreted. This becomes the basis for the decrease in cholesterol level [14,21,22]. Another mechanism is that fiber can also inhibit the reduction of HMG-CoA enzyme, thus, decrease the synthesis of cholesterol within the liver [21].

The influence of rice bran as hepatoprotective in Rats with hypercholesterolemia.

An activity of hepatoprotective of RB in rats with hypercholesterolemia can be seen based on several biochemical parameters such as, the activity of the AST and ALT enzymes which indicates the damage in the liver cells. The MDA level to see the possibility of lipid peroxidation in liver organ. Hepatoprotective activity can also be supported by the histopathology observation of the liver tissue.

These enzymes are very sensitive indicators of damage or destruction happening in the liver cells. When the liver cells are damaged such as fatty, inflammation, up to cells damage such as apoptosis or necrosis, these AST and ALT enzymes will be leaked into blood circulation due to the increase of membrane permeability. Therefore, within the blood, the level of these enzymes increases [23,24].

In Table 2, hypercholesterolemia therapy using RB with the variation of dosages of 270 mg kg^{-1} bw, 540 mg kg^{-1} bw, and 850 mg kg^{-1} bw influences the significant decrease of AST and ALT enzymes activities ($p<0.01$). Compared to the negative control group, the AST activity decreases by 29%, 40%, and 54%, while the activity of ALT enzyme decreases by 31%, 47%, and 64.88%. This decrease is due to the antioxidant effect of RB which serves in preventing the free radical, prevent oxidative stress and inhibit the fat oxidation [24].

The MDA level measurement in liver organ shown in Table 3, where administration of RB with the variation of dosages mentioned above has experienced significant reduction ($p<0.01$) compared to the positive control group with the reduction of MDA level by 46%, 63%, to 79% sequentially. RB is proven to be able to reduce MDA level due to its antioxidant content which able to capture the free radical and inhibit lipid peroxidation, hence, can reduce the free radical due to induction of high cholesterol diet.

The antioxidant content in rice bran seen in antioxidant test IC_{50} is 75.18 µg mL^{-1}. IC_{50} is a number which shows the concentration of extract (µg mL^{-1} or ppm) which can inhibit 50% of oxidation. Based on table 4 the antioxidant within the rice bran is classified as a strong antioxidant [20]. Several previous studies have shown that one of the antioxidants within the RB is Vitamin E (tocopherol/tocotrienol) [25,26].

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Table 1. Total cholesterol levels in serum of hypercholesterolemic rats

Treatment Group	Total cholesterol levels (mg/dL)	Total cholesterol levels (%)	
		Increasing	Decreasing
Negative control	173.5 ± 3.69	-	-
Positive control	247.5 ± 2.88	42	-
RB (270 g/kg^{-1} bw)	229.75 ± 2.5**	-	7
RB (540 g/kg^{-1} bw)	204.75 ± 5.9**	-	17
RB (810 g kg^{-1} bw)	193.75 ± 4.57**	-	21

The data showed the mean ± SD (n=4). The analysis shows that rice bran significantly decreases the total cholesterol level (p-value = 0.000). Note: ** showed the value of p<0.01 toward the positive control group.

Table 2. Effect of rice bran on serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzymes in hypercholesterolemic rats

Treatment Group	AST (U L^{-1})	ALT (U L^{-1})
Negative control	56.25 ± 3.5	26.00 ± 1.82
Positive control	136.25 ± 3.3	89.00 ± 2.16
RB (270 g/kg^{-1} bw)	96.75 ± 2.21**	61.00 ± 2.94**
RB (540 g/kg^{-1} bw)	81.75 ± 2.75**	47.00 ± 2.16**
RB (810 g kg^{-1} bw)	61.5 ± 2.38**	31.25 ± 2.21**

Data shows mean ± SD (n=4) the result showed that rice bran significantly decreases the AST and ALT level (p value=0.000) note: ** shows the value of p<0.01 toward the positive control group.

Table 3. MDA levels in liver organ of hypercholesterolemic rats

Treatment Group	MDA level (µg/mL)	MDA Level (%)	
		Increasing	Decreasing
Negative control	0.67 ± 0.125	-	-
Positive control	4.7 ± 0.141	596	-
RB (270 g/kg^{-1} bw)	2.5 ± 0.216**	-	46
RB (540 g/kg^{-1} bw)	1.7 ± 0.081**	-	63
RB (810 g kg^{-1} bw)	0.95 ± 0.208**	-	79

Data shows mean ± SD (n=4) the result showed that rice bran significantly decreases the MDA level (p value=0.000) note: ** shows the value of p<0.01 toward the positive control group.

Vitamin E plays a role in neutralizing the free radical by contributing to the atom hydrogen from the hydroxyl group (OH) which available in its ring structure to the free radical [27]. The existence of this antioxidant mechanism in RB helps protect the cell structure form the free radical effect, hence reduce the level of ROS which is signified by the reduction of MDA level and reduction of AST and ALT enzymes activities. This is what made the RB potential as hepatoprotective.

Table 4. The intensity value of IC_{50}

Intensity	IC_{50} value
Very Strong	< 50 mg mL^{-1}
Strong	50 – 100 mg mL^{-1}
Medium	100 – 150 mg mL^{-1}
Weak	150 – 200 mg mL^{-1}

Liver damage can be known from the increase of AST and ALT level in the positive control group which supported by the image of liver histopathology. This group shows the accumulation of lipid around hepatocyte and plenty of necrotic hepatocytes (Figure 1.G2). Meanwhile, a significant decrease of AST and ALT level in each therapy group indicates an improvement of the hepatocyte. This is proven by the figure of liver histopathology on 270 mg kg^{-1} BW therapy which shows a decrease of lipid accumulation, regardless that the necrotic hepatocytes are still exist (Figure 1.G3). In therapy group of RB with the dosage of 540 mg kg^{-1} BW, it shows a more significant decrease of lipid accumulation, there are only a few necrotic hepatocytes left (Figure 1.G4). The histopathology image of therapy group of RB with a dosage of 810 mg kg^{-1} BW shows much smaller lipid accumulation (Figure 1.G5).

The repairment of histopathology picture in liver organ due to the antioxidant from the RB, hence, can reduce the level of free radical by inhibiting the lipid peroxidation, thus, the ability of the crude fiber to reduce cholesterol level in blood [28].

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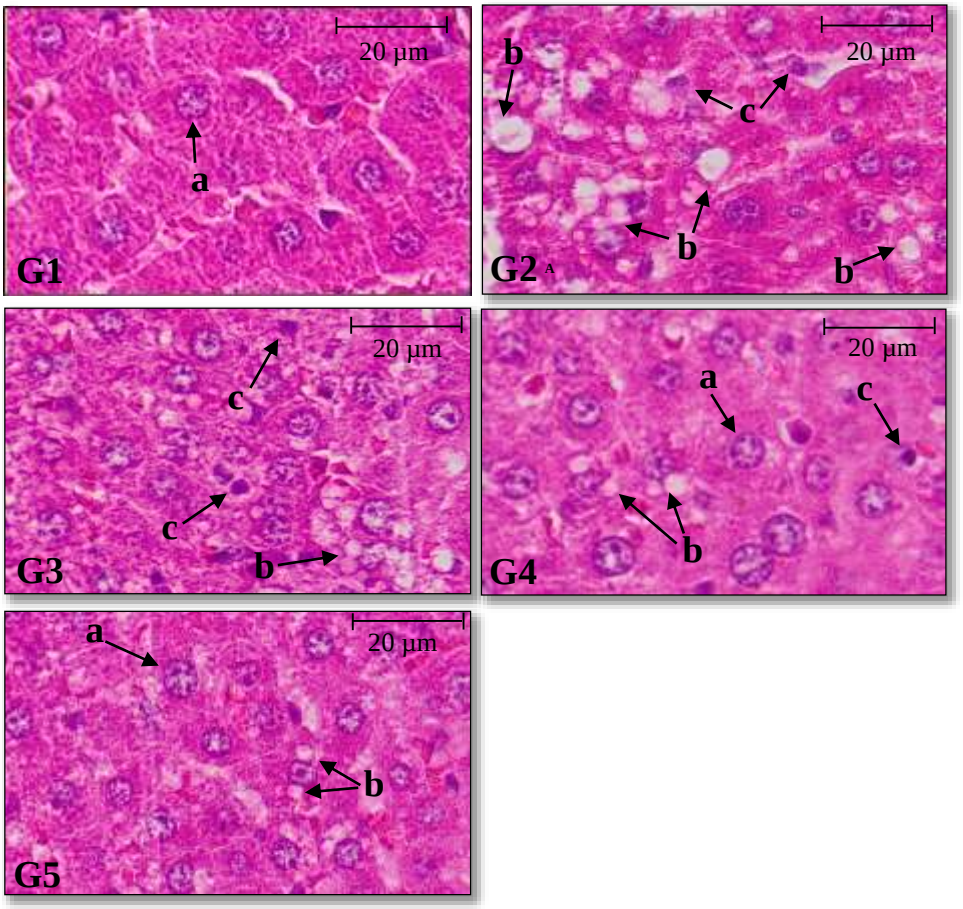

Figure 1. Histopathology Overview liver of Hypercholesterolemic Rats Using Hemotoxylin-Eosin Staining (HE) (*Magnification 400×*). (G1) negative control group, (G2) positive control group, (G3) therapy 270 mg kg^{-1} body weight, (G4) therapy 540 mg kg^{-1} bw and (G5) therapy 810 mg kg^{-1} bw. Hepatocyte (a), lipid accumulation (b), necrotic hepatocytes (c).

CONCLUSION

The conclusion of this study is that rice bran in addition to reducing the cholesterol level is also very effective in protecting the liver from free radical due to induction of high cholesterol diet, which is signified by the reduction of MDA and activity of AST and ALT enzymes.

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The Effect of Monosodium L-Glutamate (MSG) Treatment for Short and Long Terms to The Semen Quality of Adult Male Rats

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Abstract

This study was conducted to investigate the effect of MSG treatment for short and long term on the semen quality of adult male rats. Twelve male adult Wistar rats with 200-300g of body weight (BW) and 3-4 month of age were used in this study. The animals were divided randomly into 3 groups. M0 was used as a control, M1 and M2 were given with MSG 4mg/gBW for 15 and 45 days respectively. The experimental animals were sacrificed on the days 16th (to M1 group) and 46th (to M0 and M2 groups). The epididymis was isolated and semen quality (motility, viability, concentration, and abnormality of sperm) was evaluated. The results showed motility and concentration of M1 and M2 were not significantly decreased compared to M0. MSG treatment also significantly reduced viability and increased abnormality of sperm. Analysis of sperm abnormality character shows that the use of long-term MSG caused a formation of the primary abnormality (round and double head sperm) and increased the secondary abnormality (bent neck, curve tail, coiled tail, headless, and tailless) compared to control. Conclusion, semen quality decreases with consumed MSG for the long term. For this reason, reconsidering the use of MSG as an enhancer for the teste of food is very important.

Keywords: epididymis, Monosodium L-Glutamate, semen quality.

INTRODUCTION

Monosodium L-Glutamate (MSG) is a white crystal-like substance that contains 78% of glutamic acid, 22% of sodium and water [1]. The Majority, MSG used as a food additive in everyday life. MSG can increase the taste of *umami* in food and stimulated of appetite [2]. The production of MSG with the conventional method was made by the molasses fermentation process of *Micrococcus glutamicus* bacteria [3].

Glutamate given orally into the body will be absorbed by the stomach and small intestine. Then, glutamate entered the bloodstream and dissociated into sodium and L-glutamate [4]. L-glutamate then binds to the NMDA (N-Methyl-D-Aspartate) receptor in the testes. The bond between L-glutamate and NMDA receptor can open Ca^{2+} ion channels which cause Ca^{2+} influx into the intracellular and activate enzymes such as phospholipase and protein kinase. These enzymes lead to the degradation of proteins and membranes [5]. The degradation process of proteins and membranes is accompanied by the release of free radicals in the form of superoxide radical ($O2^{\cdot}$). Superoxide radical ($O2^{\cdot}$) converted to Hydrogen Peroxide (H_2O_2) by the enzyme Superoxide Dismutase (SOD). H_2O_2 will react with Fe^{2+} through an Fenton reaction to form Hydroxy Radical (OH^-) and lipid peroxidation [6]. The existence of Hydroxy Radical (OH^-) and lipid peroxidation leads transformation and fragmentation of sperm DNA, necrosis in cells, increasing abnormal of sperm, decreased sperm count [7].

Treatment of MSG at 4mg.g BW^{-1} in rats for 14 and 28 days induced alteration of sperm function in testes include significantly decrease of conversion of spermatogonia to primary spermatocytes and increase the number of inactive spermatogonia compared to controls [8]. Moreover MSG treatment in longterm can cause formation of Reactive Oxygen Species (ROS) [9]. ROS significantly promote in the level of sperm abnormality including abnormal head, normal sperm without the head, normal sperm without the tail (headless), and head without tail (tailless) [10].

In this research, we analyzed the toxic effects of treatment MSG 4mg.gBW^{-1} for 15 and 45 days on the semen quality in adult male rats. This study differs from previous studies because it uses two different duration of MSG treatment. The duration treatments are short term for 15 days and long term for 45 days. In addition, sperm abnormality parameters in this study are presented in more detail by counting the number of cells in each type of sperm abnormality.

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MATERIAL AND METHODS

Twelve male *Wistar* rats (3-4 months old and 200-350g body weight (BW) were obtained from the Animal Unit Laboratory of Gajahmada University, Yogyakarta, Indonesia. Experimental protocols were accepted by The Research Ethics Committee of Brawijaya University Malang, in accordance to the principles of Animal Care (No: 1067-KEP-UB).

Experimental animals were randomly divided into 3 groups (n = 4 per group). Control group (M0): fed standard dies and receiving mineral water; M1 group: given MSG 4mg.gBW^{-1} for 15 days, and M2 group: given MSG 4mg.gBW^{-1} for 45 days. MSG was daily administered by oral gavages. The animals were housed in conventional plastic cages measuring 39 x 30 x 12 cm (4 animals per cage) with wood shavings as bedding and maintained under standard laboratory conditions (in room temperature with a 12h/12-h light/dark cycle). Standard Food (Nova Rabbit Food, Perfect Companion Indonesia Ltd., Indonesia) and water (AQUA Golden Mississippi Indonesia Ltd.) were available ad libitum. Experimental animals were sacrificed using cervical dislocation on days 16th (M1) and 46th (M0 and M2) and the epididymis were removed and stored in buffer.

Semen Collection and Analysis

Semen were collected from right cauda epididymis. The epididymis was placed and excised in a watch glass contain 1.5 mL of NaCl 0.9% at 37°C [11]. The motility, viability, abnormality, and concentration of sperm were examined by a light microscope at 100 and 400x magnifications.

Sperm Motility (SM)

Total of 10µL semen were added to object glass. The movement of sperm at 5 microscope fields was observed by a light microscope at 100x magnification [12]. The percentage of sperm motility was recorded and scored by McMaster [13].

Table 1. Criteria of Sperm Motility Categorization by McMaster [13].

Score	Criteria
1	Very poor (0-20% progressive motile)
2	Poor (20-40% progressive motile)
3	Good (40-60% progressive motile)
4	Very good (60-80% progressive motile)
5	Excellent (80-100% progressive motile)

Sperm Viability (SV) and Abnormality (SA)

Total of 10µL liquid semen was added to object glass and was stained with 1% eosin/5% negrosin. Percentage of sperm viability and abnormality was examined by a light microscope at 400x magnification in 3 microscope fields (or up to 200 cells). This is the formulation [12,14]:

$$SV\ (\%) = \frac{\text{live sperm}}{\text{total sperm}} x\ 100\%$$

$$SA\ (\%) = \frac{\text{abnormal sperm}}{\text{total sperm}} x\ 100\%$$

Description:
SV = sperm viability
SA = sperm abnormality

Sperm Concentration (SC)

The semen was diluted 50x (20µL of liquid semen was added to 980µL of fixative solution from (1:1) NaHCO3 and formalin 10%). The spermatozoa were counted at 400x magnification using a Neubauer Hemocytometer. The number of sperm was calculated at 5 small chambers per sample using formulation (15]:

$$SC = n \times k \times DF \times 10^4$$

Description:
SC = sperm concentration was expressed as 10^6.mL^{-1},
n = number of sperm
k = number of small chamber calculated
DF = dilution factor
10^4 = room volume of hemocytometer

Statistical Analysis

The data of control and experimental group were presented as mean values and Standart Error (SE). Significant differences among treatment groups was performed using one way ANOVA with SPSS 16.0 for windows program. Result of one way ANOVA analysis is significant difference at P<0.05 and the next analyzed with *Least Significant Difference* (LSD) test.

RESULT AND DISCUSSION

The quality of semen was analyzed with the following results:

Table 2. The effects of MSG treatment on semen quality of the adult male rats

Semen Quality ± SE	Animal Group		
	M0	M1	M2
SM (%)	70.50 ± 6.85	55.00 ± 4.08	55.00 ± 10.61
SV (%)	76.57 ± 5.38	52.49 ± 8.59*	36.96 ± 6.75**
SA (%)	10.02 ± 1.62	32. 41 ± 2.25**	37.94 ± 2.21**
SC (10^6.mL^{-1})	100.63 ± 5.53	79.38 ± 13.13	69.38 ± 11.79

Notes: SM = Sperm Motility, SV = Sperm Viability, SA = Sperm Abnormality, SC = Sperm Concentration, SE = Standard Error, M0 = Control, M1 = treatment MSG for 15 days, M2 = treatment MSG for 45 days,
*(p ≤ 0,05), ** (p ≤ 0,01)

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Sperm Motility

Treatment of MSG 4mg.gBW^{-1} once daily for 15 days (M1) and 45 days (M2) no significanly reduced motility of sperm from 70.50 to 55% as compered to control group (Table 2). Although, there were no significant differences in the sperm motility between M1 and M2 but the use of MSG short and long-term induced a decrease in the percentage of sperm motility by 15.5% under the control value.

The result showed that treatment with MSG caused a decrease in sperm motility compared to control. In rats, the sperm motility value was acceptable as normal motility over 70% [16]. Reduction of sperm motility can be affected by disrupted of the spermatogenesis in testes and sperm maturation in epididymis [17,18]. The inhibition of the process of spermatogenesis was caused by a greater extent of oxidative damage in the testis. The long term (for 30 days) of MSG exposure to male rats enhance significantly of the lipid peroxidation level of the testes. The lipid peroxidation is the generation of Reactive Oxygen Species (ROS) [9]. High level of ROS in spermatozoa significantly decreased mitochondrial function and caused Adenosine Triphosphat (ATP) depletion [18]. The ATP was requeired by the sperm to mobility and capacitation process [20]. Moreover, ROS can cause the disruption of membrane fluidity. So, the ATP depletion and disruption of membrane fluidity can lead to loss of sperm motility [21]. ROS can reduce sperm motility and the ability of sperm capacitation through the pathways of membrane fluidity alteration, leading to an alterationin sperm motility parameters (22].

Sperm Viability

The sperm viability on male rats of M0 group was reduced significantly after MSG treatment (M1 and M2 groups). The highest of sperm viability was found in M0 group and the lowest in the M2 group with a reduction by 39.61% (Table 1). The treatment of MSG at longer term for 45 days caused a very significantly reduced of the sperm viability from adult male rats.

The consumption of MSG-induced the destroyer of a lipid membrane by elevated ROS [23]. The lipid membrane was composed of abundant Polyunsaturated fatty acids (PUFAs) which are the targets of ROS. ROS steal electrons from PUFAs of a cell membrane and initiating lipid peroxidation. Peroxidation of PUFAs in sperm cell membranes can give to cell dysfunction with loss of membrane function and integrity [24]. Degenerative of sperm membranes can cause reducing of membrane fluidity which makes a sperm die quickly and decrease of sperm viability [18].

Sperm Abnormality

The orally given of MSG for 15 and 45 days both induced a significant elevated of the sperm abnormality over the control group values (22.39 and 27.92%, respectively) (Table 1). The percentage of sperm abnormality (both primary and secondary abnormal) was very significantly increased ($p \leq 0.01$) in the MSG treatment group (M1 and M2) compared to the control group (Figure 1). Primary abnormal only occurred in the M2 group at 0.47%. The highest enhancement of secondary abnormal occurred in the M2 group (27.44%), followed by the M1 group (22.39%).

Sperm abnormality was divided into primary and secondary abnormality [25]. Primary abnormality is abnormality occurring during the spermatogenesis process in testes such as double head, double tail, round head, rudimentary tail and droplet cytoplasm [26]. Secondary abnormality arises during epididymal maturation, transit or ejaculation of sperm such as the bent neck, bent tail, curved tail, headless (normal sperm without head), tailless (normal sperm without tail), and coiled tail [10].

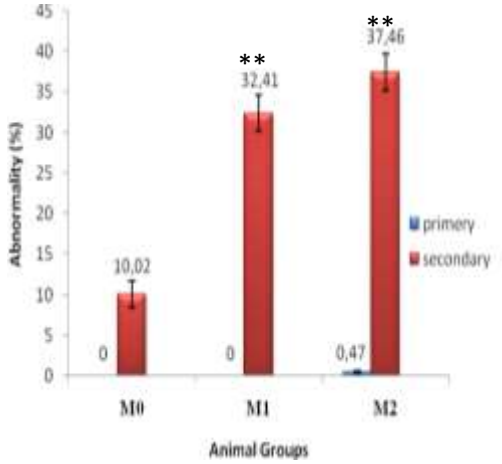

Figure 1. Effect of MSG treatment in the type of sperm abnormality. M0 (Control), M1 (treatment MSG for 15 days), M2 (treatment MSG for 45 days)

The primary abnormal was only found in the M2 group with double and round head characters (Table 3). The secondary abnormal types found in all type abnormal include the headless, bent neck, curved tail, coiled tail and tailless, while coiled tail in M2 group only. The bent neck character about 121 cells (M1 group) and 161 cells (M2 group) was the most significant

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increase as compared control. It is showed that consumption of MSG at 4 mg.gBW^{-1} dose can cause an increase of secondary abnormality and on long-term administrated of MSG lead to the formation of primary abnormality such as the double and round head characters.

Table 3. Character of sperm abnormality in male rats which treated 4mg.gBW^{-1} MSG.

AT	AP	Character	M0	M1	M2
Primary	Head	Double Head			1
		Round Head			4
Secondary	Head	Headless	52	70	71
	Mid piece	Bent neck	32	153	193
	Tail	Coiled-tail			10
		Curved tail	12	64	56
		Tailless	24	86	72
Total number of normal sperm			1097	797	669
Total number of abnormal sperm			120	373	407

Notes: AT = Abnormal Type, AP = Abnormal Position, M0 = Control, M1 = treatment MSG for 15 days, M2 = treatment MSG for 45 days.

The characteristics of sperm abnormality that occur in this research include double head, round head, bent neck, curved tail, coiled-tail, headless, and tailless. Abnormal sperm has different forms compared to normal sperm (Fig. 2). The normal sperm has a curved pointed head shape like a hook with a long straight tail (Fig. 2a). Double head sperm has 2 heads attached to one tail (Fig. 2b). The round head sperm type has a rounded head shape, not like a hook (Fig. 2c). Bent neck sperm has bent form in midpiece of sperm so the head point to approach the tail (Fig. 2d). Curved tail sperm has unstraight of the tail (Fig. 1e), the contrast to coiled-tail form has a circular tail (Fig. 2f). The headless character is normal sperm without a head (Fig. 2g), while the tailless is normal sperm without a tail (Fig. 2h).

High level of glutamate in the body can increased intracellular calcium level and cause the excessive influx of calcium ions into the mitochondria. It consequently increases the production of ROS [27]. ROS have the ability to directly attack of the purine and pyrimidine bases and degradation of sperm DNA (DNA damage). The DNA damage occured via cross-links, single and double-strand DNA breaks, and chromosomal rearrangements [28]. DNA damage during specific stages of spermatogenesis can cause inhibition of sperm maturation [18]. DNA fragmentation can inhibit the process of spermiation. It will induce the formation of primary abnormality sperm [29]. While secondary abnormality also occurs because Of hyperviscosity semen in the epididymis. Hyperviscosity caused by the functional changes of the accessory gland due to a decrease in SOD [30]. So, MSG was increase abnormal of the sperm [31].

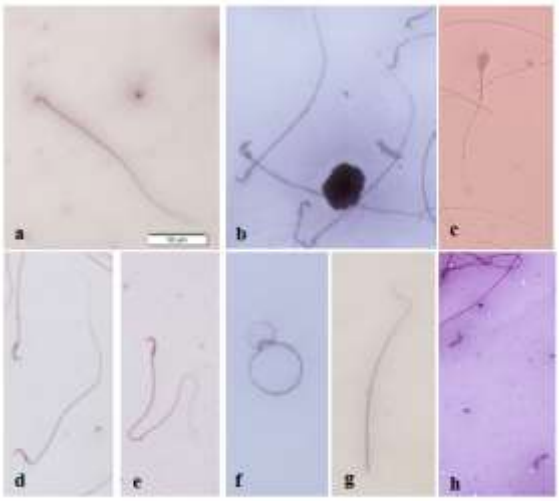

Figure 2. Effect administration of MSG in the character of sperm abormality in adult male rats. Normal sperm (a), Double head (b), Round head (c), Bent neck (d), Curved tail (e), Coiled tail (f), Headless (g), and tailless (h).

Sperm Concentration

The sperm concentration between the control and experimental group showed no significant differences ($p>0.05$). The treatment groups (M1 and M2) with orally administered of 4mg.gBW^{-1} MSG showed a reduction of sperm concentration by 21.25% and 31.25% respectively compared the control group. The use of MSG for the long term reduce sperm concentration.

The treatment of MSG could increases of Malondialdehyde (MDA) level in testes, epididymis, and accessory gland via [27,32]. MDA is the result of lipid peroxidation process which used as indicated of ROS formation [28]. The formation of ROS initiated the impairment of mitochondrial membrane fluidity, promote a membrane fusion and then decrease of ionic channels and inactivation of membrane enzymes activity [30]. Moreover, ROS can lead to DNA mutation or fragmentation, and protein oxidation. It will cause DNA damage, cell cycle irregularity, DNA repair or replication disorder and gene mutations of cells. The abnormal cell initiated the apoptotic and necrotic process [31]. The process of apoptotic and necrotic on damaged and inactive cells causes reduced sperm production in semen [32].

CONCLUSION

The results indicate that adult male rats treatment with the dose 4 mg.gBW^{-1} of MSG had deterioration of semen quality. The deterioration of semen quality involves reduced sperm motility, sperm viability, and sperm concentration. Moreover, MSG consumed in the long term was significantly increased sperm abnormality especially on the number of primary abnormal. Therefore, reconsidering the use of MSG as an enhancer for the teste of food is very important.

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The Study of Growth and Its Polyembryonic Properties of Porang Seeds (*Amorphophallus muelleri* Blume) from Various Fruit Colors in Different Planting Media

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Abstract

The ripening of Porang compound fruit *(Amorphophallus muelleri* Blume) in one cob does not occur simultaneously but moves forward from the distal region to proximal. The ripe fruit is red, almost ripe is yellow and unripe is green. The study aimed to examine the growth potential and polyembryonic properties of Porang seeds derived from red, yellow and green fruits. These seeds were grown in different media with the expectation the best plant growth can be obtained. This research was a factorial experiment which was arranged in a completely random design and each unit observation has three replication. The factor I was the source of seeds. Seeds came from various fruit colors. Each of them is red (R), yellow (Y) and green (G). Factor II is a planting medium which had 4 types, M1, M2, M3, and M4. M1 is combination of soil and husk charcoal (70%: 30%), M2 = soil: cocopeat (70%: 30%), M3 = soil: husk charcoal: cocopeat (70%: 15%: 15%) and M4 = just soil (100%). The results showed that the seeds derived from red fruit and planted on medium 2 (soil: cocopeat = 70%: 25%) potentially producing the best plant. The seeds also showed 100% polyembryony at 182 days after planting. In addition, the first, second and third shoots (originating from one seed) have different in height, canopy diameters, and petiole diameter.

Keywords: Fruitcolors, planting media, polyembriony, Porang (*Amorphophallus muelleri* Blume).

INTRODUCTION

Amorphohallus muelleri is a tuber plant of the family *Araceae*. In Indonesia, this plant is called *Porang*. A. *muelleri* contains high glucomannan. Glucomannan is widely used as a food, diet controller, cosmetics and industry [1].

The high benefits of *A. muelleri* cause an increasing demand of it from several countries such as Japan, Hong Kong, and Australia. However, production of *A. muelleri* in Indonesia, has not been able to meet this demand. The production of chips *A. muelleri* in East Java is around 600 kg – 1,000 tons, while industrial demand reaches 3,400 tons of chips [2]. Therefore, land expansion is needed and sufficiency of planting material is to be considered.

Planting material of *A. muelleri* can be obtained from tubers, bulbs and seeds. The popular one, people use tuber as planting material. In 1 hectare land area, the seeds planted from the bulbil ± 170-175 pieces and tubers ± 20-20 pieces while from the seeds can be more than ± 900 grains so that the seeds are considered the most efficient as planting material [3]. Based on observations in the porang garden Rejosari Village, District Bantur Malang, that the availability of seeds is also plenty, that in every plant A. *muelleri* can be produced ± 600-800 seeds (compounds fruit).

Germination and growth of seeds depend on the quality of the seeds. Good seeds are harvested when in the period of physiological ripe [4]. Physiological ripe time of seeds can be known through the fruit ripening, because the ripe time of seeds and fruit occurred together [5]. In previous studies, it was also reported that fruits harvested at physiological ripe produce high quality seeds in term of viability and vigority [6]. Ripening of fruit can be identified by changes in skin color of the fruit [7]. Therefore fruit skin color, in general, may be used to determine the age of seeds in it. In *A. muelleri,* based on empirical observations, the red color of fruit skin as indicator of fruit ripening does not occur simultaneously. Often found in the field, in one cob of *A. muelleri* has three fruit colors. Red in the distal area, then in the proximal direction there appears a group of yellow and green fruit.

The level of fruit maturity also affects the level of maturity of the seeds and the ability to germinate. The ripe fruit will be followed by the maturity of the seeds in it. Seeds that are harvested when physiologically matured contain enough nutrients (carbohydrates, complex fats, and proteins) to germinate optimally. However, the immature seeds contain no maximum

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nutrients [8]. In addition, the planting media also affects seed growth, where the composition of the appropriate planting media can support seed growth [9].

Seeds of *A. muelleri* have polyembryony properties, wherein one seed has more than one embryo. This is an advantage that can be utilized in the development of A. *muelleri* through seeds [3]. This study aims to determine the potential of seeds from various fruit colors (red, yellow, green) and the effect of the composition of the planting medium used on the growth of A. *muelleri*. In addition, the characteristics of the polyembryonic properties of A. *muelleri* seeds were also examined.

MATERIAL AND METHOD

Sampling of Porang Fruit

Samples used in this study were obtained from Rejosari Village, Bantur District, Malang Regency. The sample is porang fruit which has a different color on one cob which is red in distal region, yellow in the middle and green at the base/proximal parts (Fig. 1). The difference in the color of the fruit is used as a mark of different seed ages.

Figure 1. Porang Fruit Samples with Variations Skin Color

Preparation of Seed

Seeds are obtained from Porang fruit which was grouped according to the fruit skin color (green, yellow and red). Porang fruits from each color group were peeled to get seeds, then the seeds were washed and dried. The seeds used were seeds with relatively the same size, healthy and not moldy.

Media Planting Preparation

The media used for this study consisted of 4 types of planting media compositions named M1, M2, M3, and M4 (Table 1). All media were firstly steamed for 15 minutes and then dried. Furthermore, soil, cocopeat, and husk charcoal were weighed according to the composition in the treatment and mixed well. Then 80 grams of media were loaded into 5 x 15 cm polybags.

Table 1. Composition of Planting Media

Media Type	Composition (%)
Media 1	Soil 70 + husk charcoal 30
Media 2	Soil 70 + Cocopeat 30
Media 3	Soil 70+ husk charcoal15 + Cocopeat 15
Media 4	Soil 100

Seeds Planting

The seeds that have been grouped based on the color of the fruit skin were planted on a planting media according to treatment (M1-M4). Each treatment (M) consisted of 15 polybags and each treatment had 3 replications. Therefore, the total polybags of each treatment was 45.

Parameters of Growth and Germination

The observed parameters consisted of germination and growth parameters. Germination parameters included :

a) Time of shoot emergence, namely the time needed to bring out the first shoot;
b) Germination percentage. It was calculated 130 days after planting (DAP). Germination rate used formula as below:

$$\textbf{Germination } \% = \frac{number\ of\ seed\ that\ germinate}{number\ of\ seeds\ planted} \textbf{ x } 100\%$$

c) Number days (DAP) which was required to reach 60% seed germinated (uniformity of germination)

While the growth parameters included:

a) Petiole height. It was measured from the ground to the base of the lamina.
b) Petiole diamater. It was measured using digital calipers in 1 cm above the ground.
c) Canopy diameter. It was measured from one end of the canopy to the opposite end of the canopy

Data Analysis

This research was organizedusing completely randomized design (CRD) of two factors, namely the fruit color (consisting of 3 levels) and the planting medium (consisting of 4 levels). The obtained data from both the germination and growth data were analyzed using ANOVA, if the effect of fruit color and real planting media were significant, the analysed will be continued by theTukey test with a 5 % significance level.

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RESULTS AND DISCUSSION

The Germination of *A. muelleri* Blume

The results of the ANOVA test showed that the time of shoot emergence was significantly influenced by the interaction between seeds from different fruit colors and planting media. Seeds from red fruit planted in M3 media (soil, husk charcoal, and cocopeat) produced the first shoots earliest, i.e. at 70 days after planting (Table 2).

Table 2. The time that was required by shoot of *A. muelleri* to 1st emerged from red, yellow, and green seeds which grow in various medium

Fruit Color		Time of shoot emergence (DAP)
Red	M1	87.00 ± 0.01 b
	M2	94.00 ± 7.55bc
	M3	70.33 ± 0.57a
	M4	86.67 ± 6.42b
Yellow	M1	103.33 ± 1.15cde
	M2	85.33 ± 2.88b
	M3	100.33 ± 0.57cd
	M4	108.67 ± 4.04de
Green	M1	115.00 ± 6.92 e
	M2	92.33 ± 2.88 bc
	M3	111.00 ± 3.46 de
	M4	110.00 ± 1.00 de

Notes: M1 = soil and husk charcoal (70%: 30%), M2 = soil: cocopeat (70%: 30%), M3 = soil: husk charcoal: cocopeat (70%: 15%: 15%) and M4 = just soil (100%). *) for all rows of numbers followed by the same letters, it was not significantly different based on Tukey test α 0.05.

Table 2 showed that in addition to the level of maturity of seeds, planting media type also gave effect to the time of the emergence of the first shoots. Soil, husk charcoal, and cocopeat (M3) were the media components that gave the best results in this parameter. This mixture can create loose and moist of media. Media moisture is supported by cocopeat, because of its ability to bind and store water strongly [10]. Humidity is also needed in the initial phase of germination, because seeds need water before germination.

The loose of media was allegedly due to the presence of husk charcoal. Characteristics of husk charcoal are to reduce the density of the media therefore the plant roots easily penetrate the media [11]. In addition to the roots, loose media will also make the plumula penetrate the surface of the growing media smoothly. The appearance of plumula to the surface of the growing media is a sign that A. *muelleri* seeds are beginning to sprout.

In contrast with the time of shoot emergence, percentage germination at 130 DAP was only affected by the fruit color. Based on Tukey's test, the seeds are derived from red fruit has the highest percentage germination i.e. 92% ± 7.71. Then followed by seeds from yellow fruit i.e. 64.5% ± 5.86 and the lowest seed from green fruit i.e. 37.77% ± 8.20 (Table 3).

Table 3. The Effect of fruit colors on percentage germination at 130 DAP

Fruit Color	Percentage Germination (%)
Red	92.09 ± 7.71 c
Yellow	64.50 ± 5.86 b
Green	37.77 ± 8.20 a

Notes: for all rows of numbers followed by the same letters, it was not significantly different based on Tukey test α 0.05

These results showed that red seed gave the highest germination (92%) at 130 DAP. Yellow and green seeds were following. The germination rate increased with the level of fruit maturity. In this study, the level of maturity is indicated by the color of the fruit (red, yellow and green). In research of cayenne pepper varieties, Rama also reported that germination rate of chili seed reaches a maximum when physiologically mature and will decrease when it has passed the physiologically mature [12].

The next parameter is the uniformity of germination. Every seed that has a 1 cm plumule on the surface of the media was considered to be germinated. The results of the ANOVA test showed that the uniformity of germination was significantly affected by the interaction between seeds from different fruit colors and planting media. Based on Tukey test it can be seen that treatment RM3 seeds of red-skinned fruit that are grown in medium 3 (a mixture of soil, rice husk, and cocopeat) had the shortest time to the emergence of shoots up to 60% compared to other treatment that is 92 DAP (Table 4). This result is the same as the time of the first shoots. Therefore, in this study, the seeds that were able to produce the first shoots also had the shortest time in raising shoots up to 60%.

The results showed that the seeds of yellow and green-skinned fruit at the time of harvesting have not entered physiological maturity. So that uniformity of germination is lower, and the time for the first shoots to emerge and the 60% germination are longer than the seeds of the red-skinned fruit. The seeds that are harvested before reaching mature physiology, food reserve stored in the seed embryo is insufficient and not fully formed [13]. Previous studies also reported that Cayenne (*Capsicum frutescent* L.) seeds with

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different levels of maturity had a significant effect on seed germination (%) and seed germination rate[14].

Table 4. The time that was required by shoot of *A. muelleri*to 60% germination from red-, yellow-, and green seeds which grow in various medium

Treatment		Uniformity of germination (DAP)
Red	M1	99.33 ±2.30 ab
(R)	M2	114.00 ±11.53 bcd
	M3	92.33 ±2.88 a
	M4	114.67 ±5.68 bcd
Yellow	M1	125.33 ±4.04 cde
(Y)	M2	109.33 ±5.50 bc
	M3	128.33 ±1.15 cde
	M4	133.33 ±3.05 ef
Green	M1	132.33 ±7.23 ef
(G)	M2	143.33 ±7.23 f
	M3	142.67 ±6.02 f
	M4	139.33 ±1.52 ef

Notes: M1 = soil and husk charcoal (70%: 30%), M2 = soil: cocopeat (70%: 30%), M3 = soil: husk charcoal: cocopeat (70%: 15%: 15%) and M4 = just soil (100%). *) for all rows of numbers followed by the same letters, it was not significantly different based on Tukey test α 0.05

The Growth of*Amorphohallus muelleri* Blume

The ANOVA results showed that seedling growth (height and diameter of petiole and canopy) was significantly affected by the interaction between fruit color and planting media. Based on the Tukey test, the treatment that showed the best results on the growth of A. muelleri was the treatment of RM2, seed from red fruit planted in soil and cocopeat media. The treatment was able to produce the best seeds, was higher seedlings, larger petiole diameter, and wider canopy diameter compared to the other treatments (Figure 2).

The seeds of the red fruit are thought to have entered physiological maturity. So that the availability of food reserves in these seeds is sufficient to be used to grow compared with both yellow and green seeds. Food reserves sufficient seed to accelerate the growth of shoots and roots. When shoots and roots grow faster, the growth of these plants will be faster too [15].

In addition to the level of seed maturity, seedling growth is also influenced by the planting media. In this study, media 2 which is a mixture of soil media with cocopeat is the best media to support the growth *of A. muelleri* seedlings. Cocopeat is a medium that has a high water holding capacity which reaches 14.71 times its dry weight [16]. In the previous study, it was also stated that cocopeat was able to improve the physical properties of soil, which made the soil have good drainage so that it supports root development. When the plant roots are well developed, the absorption of nutrients will be optimal. These conditions can support plant growth. Similarly, in *sengon laut (Paraserianthes falcataria)* plants where planting using soil and cocopeat growing media gave the best results on the parameters of height increase, the diameter of the petiole and the number of leaves [17].

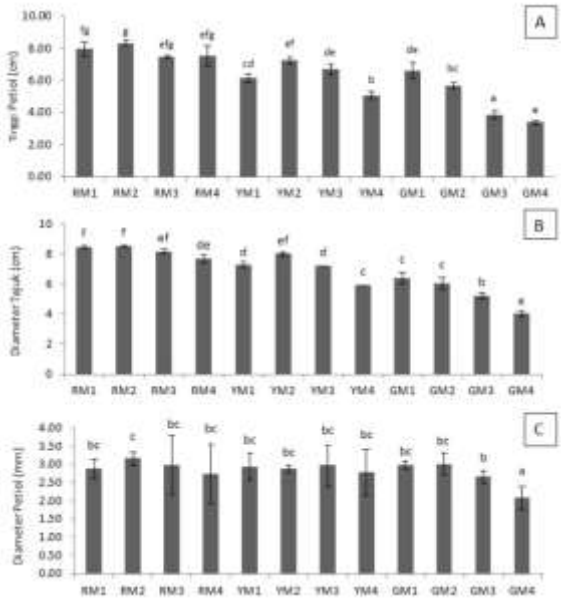

Figure 2. Interaction between Fruit Colors and Planting Media on Growth of A. *muelleri*. At 154 day after planting, A. petiole height, B. diameter of canopy C. diameter of petiole R = Seeds of red fruit, Y = Seeds of yellow fruit, G = Seeds of green fruit, M = planting medium. M1 = soil and husk charcoal (70%: 30%), M2 = soil: cocopeat (70%: 30%), M3 = soil: husk charcoal: cocopeat (70%: 15%: 15%) and M4 = just soil (100%).

Polyembryonic Properties of *Amorphohallus muelleri* Blume

Previous studies related to the germination of porang seeds, stated that in *A. muelleri* polyembryonic phenomenon was found. It is the occurrence of more than one embryo in one seed [18]. In this study, polyembryony plants were found as well (Fig. 3). Shoots produced from polyembryony seed was varied, there are 2 shoots (Fig. 3A), 3 shoots (Fig. 3B) and 4 shoots (Fig. 3C) at 182 days after planting.

The seeds of the red fruit all show polyembryonic properties, produce 2 to 4 shoots. However, the seeds of the yellow fruit, besides produce one shoot (monoembryony) also produce 2, 3 and 4 shoots (polyembryony) in all types of planting media. Production of 4 shoots

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from yellow seeds was lower than red seed. While seeds from green fruit are monoembryony and polyembryony (3 shoots) which grow in all growing media. Polyembryo seeds with three shoots only on media M3 (Table 5).

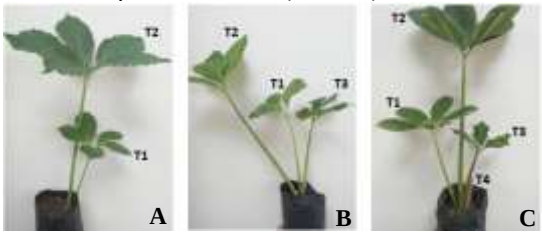

Figure 3. Plant *A. Muelleri* in 182days after planting.
A. Polyembriony plants with 2 shoots
B. Polyembryonic plants with 3 shoots
C. Polyembryonic plants with 4 shoots

Table 5. Percentage number of shoot on *A. Muelleri*

Seeds type	Planting media	Seed with variation number of shoot (%)			
		1	2	3	4
Seeds from red fruit	M1	0	6	74	20
	M2	0	30	67	3
	M3	0	30	67	3
	M4	0	17	53	30
Seeds from yellow fruit	M1	7	53	40	0
	M2	33	40	20	7
	M3	20	73	7	0
	M4	80	20	0	0
Seeds from green fruit	M1	7	93	0	0
	M2	64	46	0	0
	M3	40	20	20	0
	M4	80	20	0	0

Notes: M1 = soil and husk charcoal (70%: 30%), M2 = soil: cocopeat (70%: 30%), M3 = soil: husk charcoal: cocopeat (70%: 15%: 15%) and M4 = just soil (100%).

In this study, the first, second and third shoots that appear in one seed have different morphological characteristics (petiole, canopy diameter, and petiole). It was observed that the first shoots had an average height of 7.54 cm, the second shoots had a height of 14.7 cm (twice the height of the first shoot), the third shoots were 8.41 cm which was not significantly different from the first shoots and the fourth shoots not yet emerge leaves and petiole at 196 days after planting (Table 6).

Table 6. The Growth of polyembryonic shoots in red fruit A. *muelleri* 196 days after Planting

Type Shoot	Petiole Height (cm)	Canopydiameter (cm)	Petiole diameter (mm)
1st	7.54 ± 10.01a	8.25 ± 1.37 a	2.93 ± 0.37a
2nd	14.7± 3.34 b	12.7±2.86 b	4.53 ± 0.72b
3rd	8.41± 1.60 a	8.41±1.56 a	3.09 ± 0.39a

Notes: for all rows of numbers followed by the same letters, it was not significantly differen based on Tukey test α 0.05 in each parameter

At observations 204 days after planting, the first shoots begin to turn yellow and then wither at 210 days after planting. While the second, third and fourth shoots are still growing. At the end of the growth the second to fourth shoot have height petiol, canopy diameter and petiol diameter was not significantly different and larger than the first shoot (Table 6).

Tabel 6. Growth of Polyembryonic Shoots *A. muelleri* in 196-220 days after Planting

Type Shoot	Petiole Height (cm)	Canopy diameter (cm)	Petiole diameter (mm)
1st	7.54 ± 10.01a	8.25 ± 1.37 a	2.93 ± 0.37a
2nd	14.70 ± 1.79 b	13.69 ± 1.90 b	4.35 ± 0.52 b
3rd	14.44 ±1.90 b	13.03 ± 1.14 b	5.04 ± 0.49 b
4th	14.74 ± 4.23 b	12.70 ± 2.51 b	4.07 ± 0.72 b

Notes: for all rows of numbers followed by the same letters, it was not significantly differen based on Tukey test α 0.05 in each parameter

Polyembryo plants have more than one embryo in one seed. So that the different characteristics of the first, second and third shoots in *A. muelleri* can be possible because all three grow from different embryos in the same seed.

In the study of mangosteen fruit which is also polyembryonic, it is stated that from each bud that emerges from the same seed carries a different genetic constitution [18]. another study it was found that four of the nine polyembryonic mangosteen seeds showed differences in DNA bands in shoots that grew from the same seed [19]. This may cause differences in morphological characters between the first, second and third shoots in the same seed.

CONCLUSION

The RM3 treatment show the best results for germination, but RM2 treatment produces the best for seed and plant growth. *A. muelleri* at various level of ripening indicates the presence of polyembrionic seeds with variation on the number of shoots, where the first shoots are no larger than the second up to fourth seeds.

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Alcohol Intake Investigation of Adult Rats Based on Sperm Parameters

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Abstract

The prevalence of alcohol consumption is high among adult males during the reproductive period. The current study aimed to evaluate the impact of chronic administration ethanol on the quality of sperm in the rats. Twelve healthy Wistar male albino rats were randomly divided into 2 groups to represent the 2 phase duration. The first phase lasted 21 days and the second phase lasted 49 days. In each phase, the animals were separated into subgroups: A and B. Subgroup A represented control that received distilled water while subgroup B represented animals that received 7 $mL.kgBW^{-1}$ of 30% ethanol per day, thrice a week. The data were analyzed using ANOVA ($P<0.05$). There is no significant difference in sperm concentration and viability. However, there is a significant difference in the motility of spermatozoa between the control group and ethanol-treated group. Thus, the study indicates that ethanol administration may disturb the sperm motility and have no clear effect on its concentration and viability.

Keywords: alcohol consumption, reproductive function, sperm quality.

INTRODUCTION

Ethanol has been now reported to be among the most widely abused drug which may suppress reproductive function and sexual behavior in laboratory animals and humans. Both acute and chronic ethanol administration exerts a dual effect on the hypothalamic-pituitary-gonadal axis by directly preventing testicular steroidogenesis and by inhibiting the release of Lutenizing Hormone-Releasing Hormone (LHRH) [1].

Ethanol enlarges oxidative stress through the generation of oxygen-free radicals and lipid peroxidation on its metabolism in the body [2], mainly because ethanol administration can cut down the antioxidant cells such as Superoxide Dismutase (SOD), Catalase (CAT), and Glutathione Peroxidase (GPx). Undoubtedly, oxidative stress is one of the factors playing a key role in various pathways of alcohol-induced damage. Oxidative stress involves many conditions that stimulate either the production of Reactive Oxygen Species (ROS) or a decline in antioxidant defenses. During ethanol metabolism, the detrimental effects of ethanol not only generates a massive ROS but also form acetaldehyde. Ethanol is also oxidized by cytochrome P450 to acetaldehyde, which increases ROS, with concomitant changes in redox balances [3]. Acetaldehyde can weaken the activity in the hypothalamus with its receptors in the pituitary in the process of releasing LH and damaging the function of protein kinase C, a key enzyme in LH production [4]. Meanwhile, reactive oxygen species result in lipid peroxidation in spermatogenic cell membranes which is the cause of cell apoptosis [5].

Alcohol abuse has been considered as one of the problems associated with poor semen production and sperm quality [6]. Alcohol consumption in men has been associated with testicular atrophy [7,8], reducing in sperm count, in the percentage of motile spermatozoa and the total of spermatozoa with normal morphology [9,10]. Additionally, the negative impact of chronic alcoholism on sperm parameters was significantly decreased in semen volume, sperm count, motility and number of morphologically normal spermatozoa [7].

In rats, ethanol caused a significant rise in the tissue and plasma lipid peroxidation and decrease the tissue and plasma antioxidants such as superoxide dismutase, glutathione, catalase, peroxidase, vitamin C and vitamin E [11]. Dosumu [12] reports that administration of 30% alcohol in adult rats has made an impact on the reduction of spermatogenic cells, tubular atrophy, cross-sectional areas, and tubular diameter. Testicular weight, sperm motility and sperm count were also declined. Hormonal assay indicated a severe reduction in the levels of testosterone. Therefore, this study was undertaken to determine the effects of chronic administration of ethanol followed by abstinence on sperm parameters of adult rats.

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MATERIALS AND METHODS

Research Materials

Twelve healthy Wistar male albino rats (average weight 280-340 g) were housed in the animal holdings of the Animal Physiology Laboratory, Faculty of Mathematics and Natural Sciences, University of Brawijaya, in well ventilated plastic cages with a 12/12 h light/dark cycle at 21-24°C and free access to rodent food and water. Approval for the study was obtained from the Animal Care and Use Committee of University of Brawijaya number 1079-KEP-UB. Thirty percent (30%) of ethanol prepared from absolute ethanol (99.86%) Smart Lab (PT. Smart Lab, Indonesia).

Research Methods

Animals were randomly divided into 2 groups to represent the 2 phase duration. The first phase lasted 21 days and the second phase lasted 49 days. In the previous result [12], administration of ethanol 30% for 14 days could reduce the sperm count, motility and morphology and the intake of 21 days ethanol was considered to provide more significant results due to longer ethanol administration. The second factor was the time required for the formation of rat spermatozoa was 49 days [13]. In each phase, the animals were divided into subgroups: A and B. Subgroup A represented control that received distilled water while subgroup B represented animals that received 7 $mL.kgBW^{-1}$ of 30% ethanol per day, thrice a week [14] in details:

A1 (Control group 1) : distilled water
B1 (Treatment group 21 days) : ethanol 30%
A2 (Control group 2) : distilled water
B2 (Treatment group 49 days) : ethanol 30%

At the end of each phase, rats were sacrificed. Right after, testes and epididymis harvested for histological studies.

Sperm Analysis

The epididymis was placed in a Petri dish containing 500 µl PBS and cut into pieces using scissors to allow the spermatozoa to come out from the epididymis. Sperm quality was determined by the concentration, motility, and viability.

Sperm concentration was counted by using an improved Neubauer hemocytometer. Sperm suspensions were directly diluted 1:20 in a diluting solution of Na_2HCO_3 and formalin in distilled water. The results of dilution were then put into the counting chamber and the number of sperm was counted. Sperm motility was analyzed by taking sperm suspensions in the Petri dish using a micropipette and dropping 1 drop on the glass object. Furthermore, it was observed using a light microscope with 400x magnification. Sperm viability measurement was done by using eosin (EO) dye exclusion test. The sperm suspension was mixed with 10 µL of eosin-nigrosin dye. A thin smear of sperm was prepared after 1 min and the number of viable sperm was determined out of 200 sperm in 10 microscopic fields (400x). The live spermatozoa cells were white and the dead were stained red [14].

Statistical Analysis

The data obtained from all the groups were compiled and statistically analyzed also presented as mean ± standard deviation. Differences between groups were compared using one-way ANOVA with $p < 0.05$ considered significant and followed by Tuckey's Post hoc test.

RESULTS AND DISCUSSION

The count of spermatozoa can be seen in Table 1. The statistical test of spermatozoa concentration shows that there is no significant difference in each treatment ($p > 0.05$).

Table 1. Sperm Concentration

Treatment Group	Sperm Concentration (10^6.mL ± SD)
A1	74.72 ± 10.75
A2	91.66 ± 7.94
B1	53.61 ± 22.21*
B2	91.66 ± 7.94

Notes:
* = Significant
SD = Standard Deviation
A1 (Control group 1) : distilled water
A2 (Control group 2) : distilled water
B1 (Treatment group 21 days) : ethanol 30%
B2 (Treatment group 49 days) : ethanol 30%

The results of this study indicate a decrease in spermatozoa concentration after alcohol intake for 21 days and it reached the lowest level of the treatment. Sperm concentration in ethanol treatments for 21 days showed a lower value compared to the control group. Whereas ethanol administration for 49 days showed the same results compared to the control group.

However, there was no significant difference between all treatments. The use of ethanol for the longer term does not affect sperm count. Ethanol does not significantly affect the concentration of spermatozoa, because the metabolism of ethanol in the rat body does not

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produce excessive amounts of ROS, so oxidative stress conditions do not occur. The body's natural antioxidants can reduce free radicals so that no cell death occurs. This is supported by the previous study that explained sperm concentration, total sperm count, and percentage of spermatozoa with normal morphology were negatively associated with increasing habitual alcohol intake [15]. No statistical differences in seminal parameters found between the degrees of alcohol consumption and control [16].

Sperm Motility

There are significant differences between treatment groups ($p > 0.05$) as shown in Table 2. The table shows that treatment groups of ethanol administration both in 21 days and 49 days have sperm motility level lower than the control group. This study proves that ethanol impaired motility of spermatozoa. The percentage of sperm motility level of the control group in 21 days was 74.66% and 49 days was 77.33%. This level indicates that the control group belongs to the normal category (>50%). While the sperm level of the treatment group was 37.33% and 41.66% shows that there were subfertile category and ethanol play an important role in this [17]. A decline of sperm motility can be affected by disrupted of the spermatogenesis in testes and sperm maturation in epididymis [18].

Table 2. Sperm Motility

Treatment Group	Sperm Motility (%± SD)
A1	74.66 ± 3.51
A2	77.33 ± 2.88
B1	37.33 ± 3.05*
B2	41.66 ± 7.02*

Notes:
* = Significant
SD = Standard Deviation
A1 (Control group 1) : distilled water
A2 (Control group 2) : distilled water
B1 (Treatment group 21 days) : ethanol 30%
B2 (Treatment group 49 days) : ethanol 30%

The ethanol-related decline in spermatozoa motility in the treated groups is one of a range of indicators that chronic ethanol consumption may endanger the structural integrity of the spermatozoa through the mitochondrial pathway. Ethanol makes mitochondria less functional by carrying out mitochondrial protein synthesis [19]. This suppresses oxidative phosphorylation of spermatozoa cells [20] leading to enzyme inactivation [21]. Ultimately, this results in numerous alterations within the mitochondria may stimulate both necrotic and apoptotic cell death.

Ethanol affects mitochondrial function. Mitochondria produce ATP needed for the movement of the flagella of sperm cells. Hence, a reduced or impaired mitochondrial function will prevent sperm motility as observed in the alcohol-treated groups of this study. It reported that mitochondria are targets for oxidative stress-related signal control cell fate [22].

Sperm Viability

The percentage of spermatozoa viability shown in Table 3. There were no significant differences between the ethanol-treated group compared to the control group ($p > 0.05$). The highest level of viability of sperm was A1. This level was far from the other groups, while the lowest level was reached by B1 about 42%.

Table 3. Sperm Viability

Treatment Group	Sperm Motility (%± SD)
A1	87.76 ± 9.25
A2	61 ± 1.34
B1	42.31 ± 10.66*
B2	50.33 ± 6.88

Notes:
* = Significant
SD = Standard Deviation
A1 (Control group 1) : distilled water
A2 (Control group 2) : distilled water
B1 (Treatment group 21 days) : ethanol 30%
B2 (Treatment group 49 days) : ethanol 30%

Sperm viability in ethanol treatments for 21 days showed a lower percentage compared to the control group. As well as giving ethanol for 49 days also gave lower results than the control group. However, the decrease in the percentage of viability in the ethanol treatment groups did not show a significant difference with the control rats.

Living spermatozoa are not able to absorb color because the membrane permeability is still in good condition so that eosin-nigrosin. Dye cannot enter the cell. Conversely, eosin-nigrosin dye can enter the dead spermatozoa cells which result in cells appearing red. This is because the permeability of the membrane decreases so that dyes easily enter the cell [23].

Membrane damage occurs due to the results of ethanol metabolism that produce free radicals, which can damage membrane integrity and permeability [24]. Based on the results, 30% of ethanol did not produce excessive ROS production, which later damages the cell membrane. So that lipid peroxidation becomes

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reduced, which causes decreased sperm cell death.

Within normal physiologic conditions, internal antioxidants help to maintain a low level of oxidative stress in the semen [25]. Nevertheless, if the production of free radicals is excessive, then the body's natural antioxidants are not able to neutralize, which results in an imbalance between antioxidants and free radicals. In this case, antioxidants such as superoxide dismutase, catalase, glutathione peroxidase were thought to play a role suppresses free radicals by giving hydrogen atoms which cause neutralized ROS [26].

Percentage of survived (lives) spermatozoa was higher compared to the percentage of motility for all treatments. This is a normal condition because immobile spermatozoa do not necessarily die. Spermatozoa that only move slowly, but still alive, it won't absorb eosin color. Partodiharjo [27] suggests that the spermatozoa that not moving are not necessarily dead. So does the one that not adsorb the color, while on interpretation with the basis of moving and not moving is considered immotile.

CONCLUSION

The 30% ethanol induction did not have a sharp impact on sperm concentration and viability levels. However, the percentage of motility spermatozoa was different between the ethanol-treated group and control group. It reduces sperm motility quite significantly.

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Potential of Olive Oil Extract (*Olea europaea*) For Affecting Lipid Profile, Lipid Oxidative and Fatty Liver on Hiperlipemic Rats (*Rattus norvegicus*)

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Abstract

Olive oil (*Olea europaea*) contains 55 to 83% oleic acid which is a single chain unsaturated fatty acid or mono-unsaturated fatty acid (MUFA), and 2% phenolic components in the form of hydroxytyrosol and tyrosol. This study aims to determine the potential of olive oil extract (*Olea europaea*) in reducing cholesterol and malondialdehyde levels, along with inhibiting fatty liver development in hiperlipemic rats. Sixteen rats were divided into four groups, the first group was healthy control group, the second group was positive control group, received a high-fat diet containing 100 mg of cholesterol powder, 25 mg of cholic acid powder and 1 mL of quail egg yolk for 28 days. Third and fourth treatment groups were received for 28 days, plus 0.828 g (1 mL) and 1.656 g (2 mL) of olive oil extract daily for 14 days, respectively. The measurement of total cholesterol used cholesterol test strips based on oxidation enzyme reaction cholesterol esterase and cholesterol oxidase that produce hydrogen peroxidation, then analyzed with biosensor refractophotometry. Measurement of malondialdehyde used thiobarbituric acid (TBA) test. Histological observation of fatty liver was assessed using a NAS (Non-alcoholic fatty liver disease score). The results showed that 1.656 g (2 mL) of olive oil extract per day decreased total cholesterol level up to 44.41 %, malondialdehyde level up to 61.75%, and NAS score up to 50%, compared with positive control. It was concluded that olive oil extract was decreasing total cholesterol level, as an anti-oxidant and prevent fatty liver development.

Keywords: fatty liver, hyperlipidemia, malondialdehyde, *Olea europaea*.

INTRODUCTION

Hypercholesterolemia causes an enzymatic oxidation reaction occurs, between oxygen radicals and lipoprotein, which produces lipid radicals or LDL oxidation. Lipid hydroperoxide (ROOH) is the first stable product of a lipid peroxidation reaction [1]. Malondialdehyde (MDA) is the result of a reaction from the decomposition of lipid peroxidation. The accumulation of triglycerides in the liver, or steatosis, increases the susceptibility of hepatocytes, which are mediated by the second stage, such as inflammatory cytokines and adipokines, mitochondrial dysfunction and oxidative stress, which cause steatohepatitis and or fibrosis [2].

Olea europaea contains oleic acid and a minor phenolic component, hydroxytyrosol, which can reduce LDL cholesterol levels, by increasing triglyceride clearance. Oleic acid can also reduce LDL oxidation and the production of reactive oxygen species, which can reduce levels of malondialdehyde. Olive oil can reduce inflammation and lipid formation and inhibit the occurrence of liver steatosis [3]. This study was conducted to analyze the potential of olive oil containing oleic acid in improving lipid profiles, reduce oxidative stress, and reduce fatty liver in hiperlipidemic rats.

MATERIAL AND METHOD

Animal Preparation

The male Wistar strain (*Rattus norvegicus*), around two to three months olds, weighing between 180-200 g obtained from UPHP (Experimental Animal Development Unit), Gadjah Mada University, Yogyakarta and having received the Ethical Clearance Certificate University of Brawijaya with Number 919-KEP-UB. Rats were acclimatized for two weeks, placed in 16 cages and received standard food. Rats were divided into 4 groups, each group consisting of 4 rats. Healthy control group (K1) with a standard diet, positive control group (K2) with a high fat diet for 28 days, two treatment groups (P1) and (P2), with a high-fat diet for 28 days plus olive oil extract of 0.858 g (1 mL) and 1.656 g (2 mL) every day, for 14 days.

The composition of a High-Fat Diet

A high-fat diet consists of 100 mg of pure high-grade cholesterol powder Bioworld brand, 25 mg of cholic acid powder from the Tokyo Chemical Japan Industry brand and 1 mL of egg

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yolk, coupled with water to a solution of 3 mL. Based on the composition of the hyper cholesterol induction diet which consisting of 1% cholesterol powder, 0.25% bile salt or cholic acid and 4% animal fat, for 21 days [4].

Determination of Dosage of Olive Oil Extract

Bertoli extra virgin olive oil, produced by Carapelli Florence, Italy, the dosage of olive oil to reduce the risk of cardiovascular disease, is 2 tablespoons or 23 g per day [5]. Dosage was converted between species, from human doses, with a human body weight of 70 kg, equivalent to 200 g of rat body weight, obtained 0.018, based on Laurence formula. The dose of 2 tablespoons or 23 g is converted to rats weighing 200 g, 23 times multiplied by 0.018 resulting in 0.414 g or 0.5 mL per day. Giving olive oil, in the treatment groups 3 and 4, with a dose of 0.828 g (1 mL) and 1.656 g (2 mL) every day, for 14 days.

Analysis of Oleic Acid in Olive Oil, Using Liquid Chromatography-Mass Spectrometry, Electro Spray Ionization (LC-MS-ESI)

Total of 3 mL olive oil inserted into the liquid chromatography solvent reservoir, will separate the oleic acid compound with other compounds, based on differences in composition and molecular weight. The oleic acid compound is converted into an electrically charged molecular ion, to calculate its molecular mass, into a chromatogram.

Examination of Total Cholesterol Levels

Total cholesterol levels were measured using the easy touch, cholesterol test strips based on the presence of the cholesterol esterase enzyme oxidation reaction and cholesterol oxidase which produced hydrogen peroxidation, then analyzed by biosensor reflectophotometry.

Examination of Malondialdehyde (MDA) Levels

Measurement of MDA levels was carried out with the Bioassay System Quantichrom TBARS Assay Kit (DTBA-100), based on the formation of a reaction between malondialdehyde and thiobarbituric acid, which produces pink fluorescence at an intensity of 560 nm. Serum for the examination of malondialdehyde, obtained from blood taken through the heart using 10 mL syringe as much as 10 mL, then put into vacutainer. Total of 5 mL blood is stored at room temperature for two to ten hours, then centrifuged at 2000 rpm for 20 minutes, then stored at -40^{o}C.

Histological Preparations and Liver Histology Examination

The liver organ is taken and cut into a size of 1 x 1 cm and then fixed in neutral buffer formalin (NBF) of 10% for 24 hours. Tissue cassettes are included in the tissue processor for the stages of dehydration, clearing, embedding. After blocking with paraffin, cut with a microtome in a thickness of 5-6 microns. Furthermore, the preparations were stained with staining of hematoxylin-eosin (HE).

Calculation of Non-Alcoholic Fatty Liver Disease Score (NAS) Score

The calculation was conducted based on the histology of liver tissue in the form of steatosis, hepatocyte ballooning, and inflammation. Observations with microscopes were seen in twenty view fields with 200 times magnification. The NAS score is between zero and eight, based on the amount of steatosis, ballooning, and inflammation.

Data Analysis

This study was tested using ANOVA with a confidence level of 95%, every treatment was replicated four times, followed by the test Tukey's real difference with a confidence level of 95%.

RESULTS AND DISCUSSION

The Presence of Oleic Acid in the Olive Oil Extract

Analysis of extra virgin olive oil using MS-ESI LC, there is an ion mass per charge ($m.z^{-1}$) with a range of 280.50 - 281.50 $m.z^{-1}$ which corresponds to the mass of ions per electronic charge of oleic acid (Fig. 1A). Based on data from Mass Bank Records MT000029 that used LC-MS-ESI, the ion mass per electronic charge ($m.z^{-1}$) of oleic acid is 281.3 $m.z^{-1}$.

Besides that, there is an ion mass per charge ($m.z^{-1}$), with a range of 254.50 - 255.50 $m.z^{-1}$, and 278.50 - 279.50 $m.z^{-1}$, which corresponds to the mass of ions per charge of palmitic acid and linoleic acid (Fig. 1B and 1C). Based on Mass Bank Record data MT000114, ion mass per electronic charge ($m.z^{-1}$) of palmitic acid and linoleic acid using MS-ESI LC-255.3 $m.z^{-1}$and 279.3 $m.z^{-1}$. In addition to these three types of fatty acids, there is an ion mass per electronic charge ($m.z^{-1}$) in the range 298.50 - 299.50 $m.z^{-1}$ which corresponds to the ion mass of tyrosol glucoside, which is a phenolic component of olive oil (Fig. 1D).

So that it can be concluded, from the examination of Bertolli extra virgin olive oil, using

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LC-MS - ESI, there are oleic acid, palmitic acid and linoleic acid which are the three most fatty acids in olive oil.

Oleic acid is a monounsaturated fatty acid (MUFA), while palmitic acid is a type of saturated fatty acid, and linoleic acid is a double chain unsaturated fatty acid (PUFA). In addition to fatty acids, the phenolic component is tyrosol glucoside, which is a derivative of tyrosol, where tyrosol is a major substance from the phenolic component.

Every 100 g of olive oil contains fatty acids, namely 73.7 g of oleic acid which includes monounsaturated fatty acids (MUFA), 13.5 g of palmitic acid which is a saturated fatty acid, 7.9 g of linoleic acid and alpha-linolenic acid, which is polyunsaturated fatty acid (PUFA) [6].

Olive Oil Extract for 14 Days Decrease Total Cholesterol Levels

The administration of 0.828 g of olive oil extract (1 mL) and 1.656 g (2 mL) daily for 14 days in P1 and P2, significantly reduced ($p < 0.05$) cholesterol levels, compared with the positive control group. Total cholesterol levels in the group of P1 and P2 were 99 $mg.dL^{-1}$ and 94.5 $mg.dL^{-1}$, decreased 41.76% and 44.41% compared to the positive control group (Table 1).

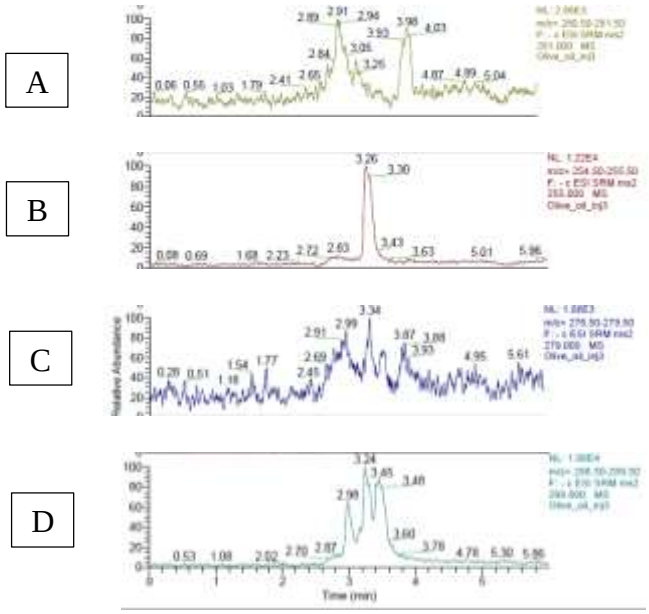

Figure 1. Ion Mass per Charge of Fatty Acids and Phenolic Components in the Extract Olive Oil
A. Ion mass per charge of oleic acid
B. Ion mass per charge of palmitic acid
C. Ion mass per charge of linoleic acid
D. Ion mass per charge of tyrosol glucosi

Table 1. Total Cholesterol Levels of Mice After a 28-day High-fat Diet and Oil Olives Extract for 14 days

Group	Total Cholesterol ($mg.dL^{-1}$)		
	High fat diet for 14 days	High fat diet for 28 days + olive oil extract for 14 days	Decrease of total cholesterol Compared with positive control (%)
K1	51.25 ± 0.95^{a}	49.25 ± 0.95^{a}	-
K2	176.54 ± 3.41^{b}	170.00 ± 10.8^{c}	-
P1	177.00 ± 2.16^{b}	99.00 ± 0.81^{b}	41.76
P2	173.00 ± 6.21^{b}	94.50 ± 4.50^{b}	44.41

Notes: K1 = Healthy control, K2 = Positive control, high fat diet for 28 days, P1 = a high fat diet for 28 days + olive oil extract 0.828 g (1 mL) per day for 14 days, and P2 = high fat diet for 28 days + olive oil extract 1.656 g (2 mL) per day for 14 days

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The decrease of cholesterol levels in the P1 and P2 group caused by the content of oleic acid and tyrosol in olive oil extract detected by LC-MS-ESI. Olive oil extract containing oleic acid and phenolic components can increase HDL capacity to accelerate the removal of cholesterol from macrophages by increasing the expression of adenosine triphosphate (ATP)-binding membrane cassette system (ABCA1 and ABCG1) [7]. Oleic acid can reduce HDL oxidation in humans, so it can increase the disposal of cholesterol from macrophages because HDL oxidation can damage HDL function and reduce the ability of HDL to eliminate cholesterol from macrophages [8]. Olive oil phenolic compounds can increase the size and stability of HDL, which can reduce triglycerides, and reduce HDL oxidation by increasing polyphenol metabolism in a lipoprotein [9].

Olive Oil Extract Decrease MDA Levels

The administration of 0.828 g of olive oil extract (1 mL) per day and 1.656 g (2 mL) daily for 14 days in P1 and P2 group, significantly reduced ($p < 0.05$) MDA levels, compared to the positive control group.

The average MDA levels in the P1 and P2 group were 8.07 $\mu mol.L^{-1}$ and 7.41 $\mu mol.L^{-1}$, decreased 58.29% and 61.75%, compared to the positive control group (Fig. 3, Table 2). Giving a high fat diet plus olive oil extract 0.828 g (1 mL) and 1.656 g (2 mL) every day for 14 days in P1 and P2 group, significantly reduced MDA levels, because olive oil contains oleic acid and phenolic components which can inhibit LDL lipoprotein oxidation process, which results in a decrease in MDA value.

Olive oil can reduce lipid peroxidation, in fresh meat put into gastric simulation media with a pH of 3, thereby reducing levels of malondialdehyde, from 121.7 ± 3.1 $\mu mol.L^{-1}$ to 48.2 ± 1.3 $\mu mol.L^{-1}$. Oleic acid in olive oil can reduce the release of hydrogen atoms so that oxygen does not occur, which inhibits the occurrence of lipid oxidation [10].

Olive Oil Extract Decrease Non-Alcoholic Fatty Liver Disease Activity Score (NAS)

The NAS score or the Non-Alcoholic Fatty Liver Disease Activity Score is a histological assessment based on the presence of steatosis, lobular inflammation, and ballooning of hepatocytes, which describes the presence of active injury in the liver. The NAS score is from 0 to 8, which is the sum of the number of steatosis, inflammation of the liver lobe and ballooning of liver cells that occur (Table 3) [11].

The administration of olive oil extracts for 14 days at a dose of 0.828 g (1 mL) and 1.656 g (2 mL) per day, in the P1 and P2 group, decreased the Non-Alcoholic Fatty Liver Disease Activity Score (NAS), when compared to the positive group. The P1, P2 group with an average NAS score of 2.5 and 1.5, reduced NAS score of 16% and 50% lower than the positive control group (Table 4). Giving olive oil extract as much as 1.656 g (2 mL) every day for 14 days in P2 group can reduce 50% NAS scores, compared to the positive control group, because olive oil extract can reduce the accumulation of triglycerides in the liver, accelerating recovery of liver steatosis, slow fibrosis growth and prevent oxidative stress in the liver. Tyrosol which is a polyphenol, act as an anti-inflammatory in the liver [12]. Olive oil can increase the formation of anti-oxidation enzymes; improve liver tissue, by repairing hepatocyte cell membranes [13].

Table 2. Levels of Malondialdehyde after a 28-day High-fat Diet and Olive Oil Extract for 14 days

Group	MDA level average ($\mu mol.L^{-1}$)	Increase of MDA level compared with healthy control (%)	Decrease of MDA level compared with positive control (%)
K1	6,25 ± 0,73[a]	-	-
K2	19,35 ± 2,78[b]	209	-
P1	8,07 ± 1,63[a]	29,12	58,29
P2	7,41 ± 0,42[a]	18,56	61,75

Notes: K1 = Healthy control, K2 = Positive control, high fat diet for 14 days, P1 = a high fat diet for 28 days + olives oil extract 0.828 g (1 mL) per day for 14 days, and P2 = high fat diet for 28 days + olive oil extract 1.656 g (2 mL) per day for 14 days

Table 3. Stadium of Non-Alcohol Fatty Liver Disease (NAFLD), based on NAFLD activity score (NAS)

NAS	Steatosis	Ballooning	Lobular Inflammation
0	<5% (0)	None (0)	None (0)
3	5-33% (1)	Rare or few (1)	1-2 focci per 20 x field (1)
6	34-66% (2)	Many (2)	2-4 focci per 20 x field (2)
8	> 66% (3)	Many (2)	> 4 focci per 20 x field (3)

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Table 4. NAS Score After a High-fat Diet for 28 and Olive Oil Extracts for 14 days

Group	NAS score average	Increase NAS score compare with healthy control (%)	Decrease NAS score compare with positive control (%)
K1	$1 \pm 0{,}81^{a}$	-	-
K2	3 ± 0^{c}	200	-
P1	$2{,}5 \pm 0{,}57^{b,c}$	150	16
P2	$1{,}5 \pm 0{,}57^{a,b}$	33,33	50

Notes: K1 = Healthy control, K2 = Positive control, high fat diet for 14 days, P1 = a high fat diet for 28 days + olives oil extract 0.828 g (1 mL) per day for 14 days, and P2 = high fat diet for 28 days + olive oil extract 1.656 g (2 mL) per day for 14 days. NAS scores ranged 0-8, i.e. the sum of many steatosis (0-3), ballooning hepatocytes (0-2), and hepatocyte inflammation (0-3) [11].

In this study, the healthy control group (normal hepatocytes) showed in green arrows (Fig. 5A). In the positive control group, many inflammations of the lobules hepatocytes containing infiltrates (orange arrow) (Fig. 5B). In the P1 and P2 group, inflammatory hepatocytes (orange arrows), was found less than the positive control group and a few of ballooning hepatocytes (yellow arrows) (Fig. 5C) and (Fig. 5D).

CONCLUSION

The results showed that olive oil extract 1.656 g (2 mL) per day decreased total cholesterol level up to 44.41%, MDA level up to 61.75%, and NAS score up to 50%, compared to the positive control. It was concluded that oleic acid and tyrosol in the olive oil extract can decrease total cholesterol level, thereby reducing lipid oxidation and inhibiting fatty liver development.

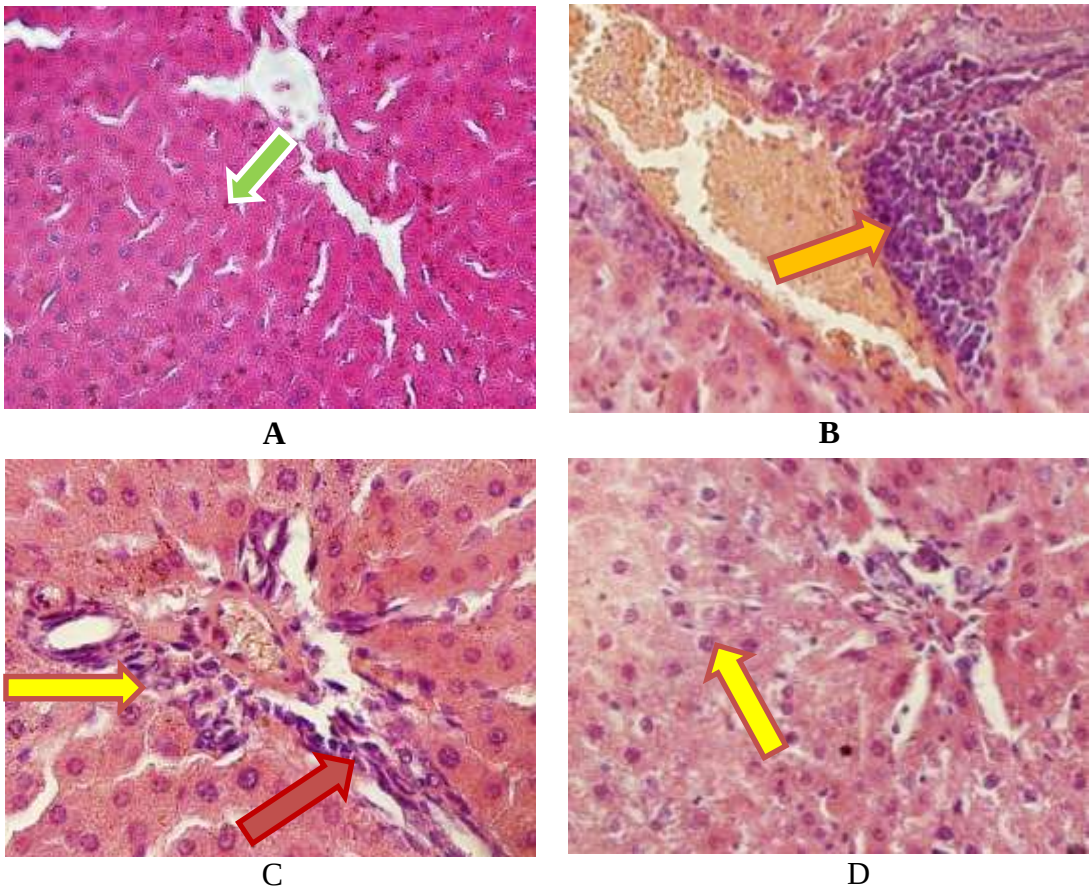

Figure 5. Hepatocyte Cells Histology.

Description:

(A) Hepatocyte cells of the healthy control group, shows there are normal hepatocytes (green arrow),

(B) Hepatocyte cell of the positive control group, with 28-day high-fat diet, shows there are many inflammation hepatocytes and infiltrates containing polymorphonuclear cells (orange arrows).

(C) Hepatocyte cells of the P1 group, with a high-fat diet for 28 days and olive oil extract 0.828 g per day (1 mL per day) shows there are hepatocytes inflammation (orange arrows) and ballooning hepatocytes (yellow arrow).

(D) Hepatocyte cells of the P2 group , with a high-fat diet for 28 days and olive oil extract 1.656 g per day (2 mL per day) shows there are few ballooning hepatocytes (yellow arrow).

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Heavy Metal (Pb) and Its Bioaccumulation in Red Algae (*Gracilaria* sp.) At Kupang Village, Jabon Sub-District, Sidoarjo District

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Abstract

Seaweeds have an inherent capacity to absorb heavy metals from marine water. This intrinsic ability allows these organisms to accumulate much amount of heavy metals over time. This study conducted at Kupang Village, Jabon Sub-District, Sidoarjo District, where almost 40% of the area consists of *Gracilaria* sp. ponds cultured. The purpose of this study was to analyze the Pb heavy metals concentration in water culture and its concentration in holdfast and thallus of *Gracilaria* sp. In this research, determination of samples in each pond was taken on day 0 (before planting), 20 days (Initial Production) and on day 40th (Post/Harvest). The aquatic parameters include salinity, temperature, acidity (pH), and dissolved oxygen (DO). Quantitative determination of heavy metals on sample using Atomic Absorption Spectrophotometry (AAS). Determinations of heavy metal accumulation in organism using Bioconcentratin factor (BCF) and Translocation factor was used to calculate the Pb heavy metal translocation process from the base to the tip of *Gracilaria* sp. The results of the study showed the highest Pb concentration found in the second sampling age 20 days, in pond 1 with 7.61 ± 0.18 $mg.kg^{-1}$, and pond 2 was 5.35 ± 0.09 $mg.kg^{-1}$. This concentration has not exceeded the threshold value that might have an effect if more than 8.6 $mg.kg^{-1}$. The highest Pb level at the holdfast of *Gracilaria* sp. found at age 0 days before planting, which is 3.38 ± 0.23 $mg.kg^{-1}$ and decreases to post-harvest (age of 40 days) which is 0.84 ± 0.00 $mg.kg^{-1}$. The Transaction Factor (TF) of Pb heavy metal value from holdfast to thallus is 1,015 thus *Gracilaria* sp. absorbs heavy metals in high concentrations at the beginning of planting and is able to release it again before harvest time.

Keywords: Bioaccumulation, *Gracillaria* sp., Heavy Metal, Histological, Red Algae.

INTRODUCTION

Red seaweed *Gracilaria changi* is a good potential source of β-carotene due to the established high content of 5.2 $mg.100g^{-1}$, which is in comparison with 6.8 $mg.100g^{-1}$ in carrots [1]. Meanwhile, *Kappaphycus alvarezii* is commercially important of red algae as a phycocolloid that is extensively applied as a thickening and stabilizing agent in food, pharmaceutical, and cosmetic industries and needed for its cell wall polysaccharide. Also, it has been used in health beverages and anticancer nutraceutical because of its antioxidant content and other nutritive compounds [2].

Heavy metals are significant environmental pollutants and their toxicity is a problem of increasing significance for ecological, evolutionary, nutritional and environmental reasons [3,4]. The most commonly found heavy metals in water include arsenic, cadmium, chromium, copper, lead, nickel, and zinc, all of which cause risks for human health if consumed usually through food [5].

Kupang Village is one of the villages located in Jabon Sub-District, Sidoarjo District, where almost 40% of the area consists of ponds. One of the leading cultivation in the village is *Gracilaria* sp. seaweed. The presence of airflow from the Porong River is known to contain heavy metals into the aquaculture environment of *Gracilaria* sp. The previous study stated that the increased lead (Pb) over the threshold in the Porong River is quite dangerous for biota [6]. Also, the results of heavy metal measurements carried out at the Porong river estuary proved that heavy metal lead (Pb) is at the threshold set by the Government with a Pb content of 0.0648 ppm [7].

Seaweeds have an inherent capacity to absorb heavy metals from marine water. This intrinsic ability allows these organisms to accumulate much amount of heavy metals over time. Previous investigations had observed the accumulation of the selected heavy metals in the different species of seaweeds. The study showed that five species of seaweeds have accumulated seven types of heavy metals (Cd, Cu, Mn, Ni, Pb, Zi, and Hg) at varying concentrations [8].

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The absorption (accumulation) process also influenced by the form of the thallus, e.g. at the base and branching of *Gracilaria* sp. Naturally, the thallus that located at the base part which attached itself to the substrate has different absorption process from the tip thallus *Gracilaria* sp. [9]. The purpose of this study was to analyze the accumulator potential of *Gracilaria* sp through determinations of the concentration and effect of Pb heavy metals on the holdfast and thallus of the talus seaweed tissue.

MATERIAL AND METHOD

This research was conducted in July-September 2018, where sampling was carried out in Kupang Village, JabonSub-district, Sidoarjo. Sampling of *Gracilaria* sp. seaweed conducted in 2 (two) ponds. From each pond, the best quality seaweed was taken as much as 1 kg. Determination of samples in each pond was taken on day 0 (before planting), 20 days (Initial Production) and on day 40th (Post/Harvest).

Aquatic Parameter

Aquatic parameters observed directly on *Gracilaria* sp. ponds. The aquatic parameters include salinity, temperature, acidity (pH), and dissolved oxygen (DO) [10].

Atomic Absorption Spectrophotometry (AAS)

Atomic Absorption Spectrophotometry (AAS) is an analytical technique that measures the concentration of elements contained in the *Gracilaria* sp sample. It is the basis for the quantitative determination of metals by using Atomic Absorption Spectrophotometry [11,12].

Data Analysis

Data obtained from the results of the study were analyzed descriptively quantitatively. AAS was used to determine the content of heavy metal lead (Pb) and Bioconcentratin factor (BCF) to calculate the value of heavy metal accumulation in organisms with the formula:

$$BCF = \frac{C_{org}}{C_{water}}$$

Description:
BCF = Bioconcentratin factor,
$C_{organisme}$ = metal content in organisms (mg.kg^{-1})
C_{water} = metal content in water (mg.kg^{-1}).
Category of BCF values [11]:
> 1000 = high accumulative properties category
100-1000 = medium accumulative properties
< 100 = low accumulative properties

Translocation factor was used to calculate the Pb heavy metal translocation process from the base to the tip of *Gracilaria* sp. [12], by the formula:

$$TF = \frac{BCF_{thallus}}{BCF_{holdfast}}$$

Categories:
TF > 1 = Phytoextraction mechanism
TF < 1 = Phytostabilization mechanism

RESULT AND DISCUSSION

Aquatic parameter

The parameters of the water sample which measurements are made directly in the field can be seen in Table 1. The results of salinity measurements at the research station exceeded the SNI 7904: 2013 threshold of 15-30‰ [13]. One of the factors that cause salinity levels increases because the sampling conducted in the dry season (July-August), so that the salt content increases due to evaporation. However, *Gracillaria* sp. has the ability to tolerate high salinity levels [14].

Table 1. Water Quality in *Gracilaria* sp. Sampling Water

Parameter	*Gracilaria* sp. production age					
	Pond 1 (Days)			Pond 2 (Days)		
	0	20	40	0	20	40
Salinity (‰)	33	35	41	33	36	35
Temperature (°C)	27	25	25	27	24	25
pH	7.5	6.8	6.9	7.5	7.3	7.0
DO (mg.L^{-1})	5	8	8	8	11	5

The results of temperature measurements on pond 1 and pond 2 of *Gracilaria* sp. in Kupang Village, is still in the range of SNI 7904; 2013 [13], i.e. 20-28°C; which is still considered good for the growth of *Gracilaria* sp. Thus, it can improve the process of nutrients absorption and accelerate the growth rate of seaweed.

Measurement of the degree of acidity (pH) in the *Gracilaria* sp. pond range for Pond 1 is 6.8-7.5 and in Pond 2 is 7.0-7.5. The pH range on Pond 1 and Pond 2 is still in the range of SNI 7578: 2010 [15], namely 6.8-8.2. Waters with a pH value = 7 are neutral, pH < 7 is said to be an acidic water condition [16]. Decreasing pH in Pond 1 which less than 7 and said to be acidic will cause greater heavy metal toxicity. The decrease in pH in the waters can also cause greater levels of pollutant bioaccumulation in organisms [17].

Dissolved oxygen content in the study location both on Pond 1 and Pond 2 has not exceeded the threshold determined by SNI 7578: 2010 [15] which is > 3.0 mg.L^{-1}. The DO content which is always above 3.0 mg.L^{-1}, can be due to research conducted in the morning before noon

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where photosynthesis takes place at that time. It causes the addition of oxygen through photosynthetic processes and the exchange of gas between water and air which causes relative dissolved oxygen levels higher [18,19].

Pb Metal Content

In Water

The Pb concentration analysis on *Gracilaria* sp. water sampling using the AAS method showed in Figure 1. In the first, second, and third water samples, the highest concentration of Pb found in water sampling before planting on Pond 1 and Pond 2, and then there was a decrease in the second and third sampling. Pb heavy metals in the waters were found in dissolved and suspended form. Lead solubility in water was quite low, so the levels were relatively small [20]. Furthermore, there was a decrease in ponds aged 40 days, which were below the threshold value of 0.09 mg.L^{-1}, allegedly exchanging lead into sediments through binding to organic matter [21].

In the Sediments

The level of heavy metals Pb in the sediment of ponds with *Gracilaria* sp. using AAS, seen in Figure 2. The highest Pb content found in the second sampling, age 20 days in pond 1 was 7.61 ± 0.18 mg.kg^{-1} and in the second pond was 5.35 ± 0.09 mg.kg^{-1}. This concentration may be caused by human activity around this area. Various sources of heavy metals include soil erosion, natural weathering of the earth's crust, mining, industrial effluents, urban runoff, sewage discharge, insect or disease control agents applied to crops, and many others [22]. This concentration has not exceeded the threshold value that might have an effect of more than 8.6 mg.kg^{-1} [23]. It means that the concentration of Pb is not too dangerous for living things.

In Holdfast of Gracilaria sp.

Measurement of Pb content in the holdfast of *Gracilaria* sp. in Kupang Village, using AAS method, can be seen in Figure 3. The highest Pb level in the holdfast of *Gracilaria* sp. found at age 0 days before planting, 3.38 ± 0.23 mg.L^{-1}, and then it decreased to post-harvest age of 40 days which is 0.84 ± 0.00 mg.L^{-1}.

Gracilaria sp. before planting is the seedlings that originated from the same aquaculture pond in the form of cuttings selected from fresh plants [24], thus experiencing stress [25] and experiencing heavy Pb heavy metal absorption. Pb Content in Pond 1 at 20 days decreased more than Pond 2. It is due to in the times of adaptation and at the age of 20 days, the Pb absorption process in *Gracilaria* sp. was very strong. The nutrients for breeding and Pb will also be absorbed.

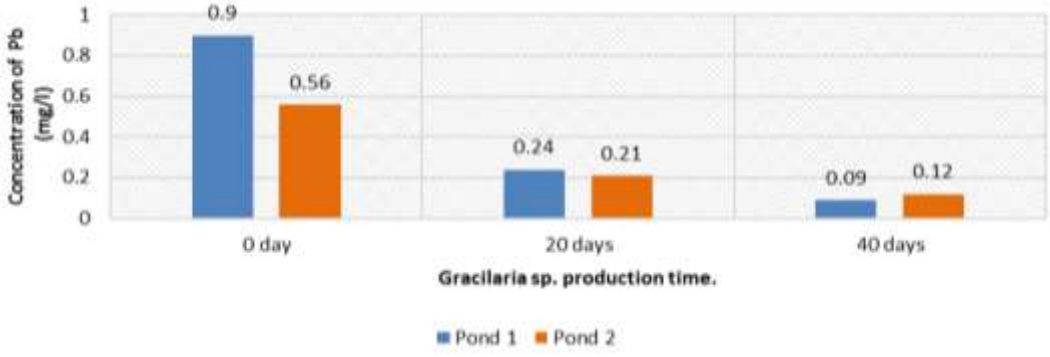

Figure 1. Pb Concentration in Water

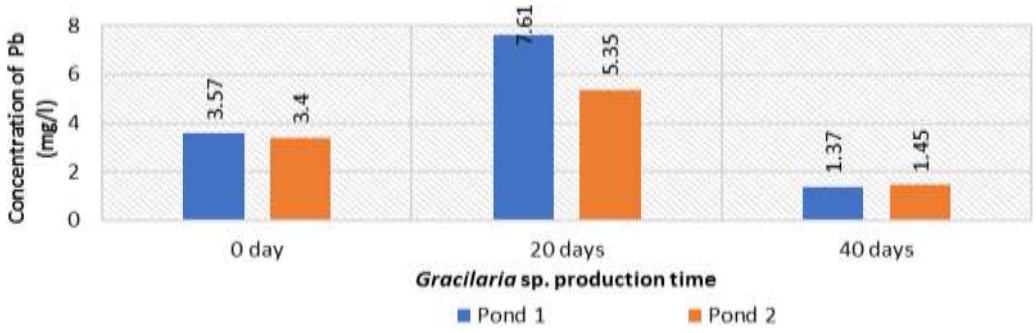

Figure 2. Pb Concentration in Sediment

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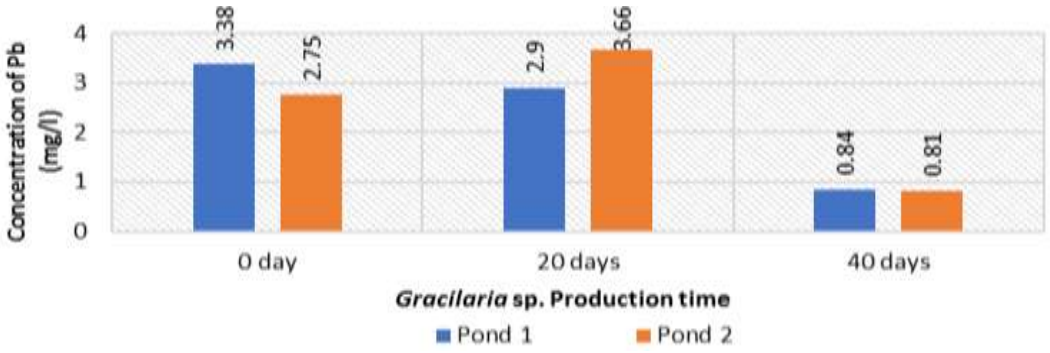

Figure 3. Pb Concentration in holdfast of *Gracilaria* sp.

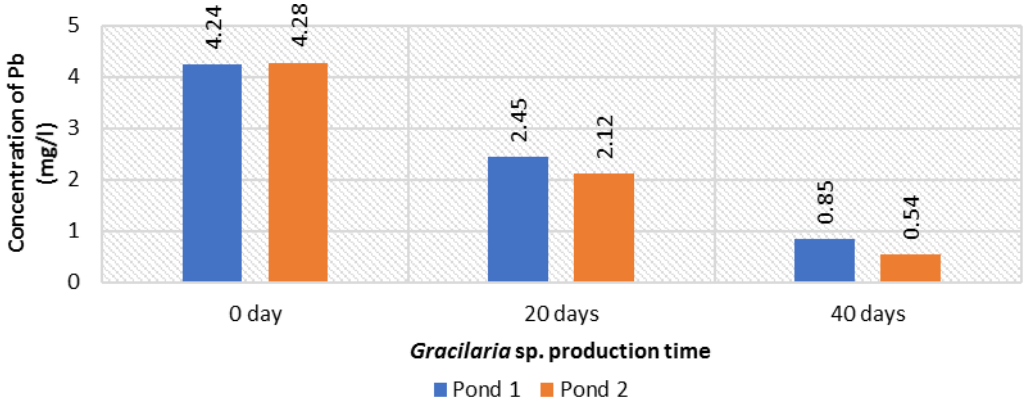

Figure 4. Pb Concentration in the Young Thallus of *Gracilaria* sp.

The high adaptation ability of *Gracilaria* sp. to change the water quality makes it able to survive in the stress of heavy metals without disrupting the growth rate. The growth rate of *Gracilaria* sp. at the end of the cell meristem is higher than the base. It is because, at the end of the cell, the branching is large, small and still young, while at the base consists of cells that are old with a larger but smaller number of branches [26].

The results of the analysis using AAS at the young thallus of *Gracilaria* sp. showed high Pb levels at the first sampling at the age of 0 days for 4.24 $mg.L^{-1}$. It then decreased at the age of 20 days to 2.12 $mg.L^{-1}$ and age 40 days to 0.54 $mg.L^{-1}$ (Fig. 4).

The process of Pb absorption by *Gracilaria* sp. influenced by the shape of the thallus. The shape of the *Gracilaria* thallus is cylindrical and forms clumps with irregular branching types. Otherwise, it does not shape like that at the base, because it has old tissue properties, thus it does not have the activity to multiply itself [27]. Material that has smaller size in diameter has a higher absorption rate than a larger diameter adsorbent [28].

From the results of observations and measurements of Pb heavy metal concentrations at the holdfast and thallus of *Gracilaria* sp., it can be used as a bioaccumulator; as well as histology at the holdfast and thallus of *Gracilaria* sp. can also be used as an accumulator. Parts of seaweed in general consist of the holdfast, which is the basic part of seaweed with functions to stick to the substrate, and thallus which is a form of seaweed growth that resembles branching [29].

Bioconcentration factor (*BCF*)

In Water and Sediment

The bioconcentration of Pb heavy metals in water and sediment are shown in Table 2. The results of the sediment BCF calculation in the water with *Gracilaria* sp. treatment age 0 days, 20 days and 40 days included in the category of low accumulation group.

It was also supported by using the t- test analysis, where significance value (t-tailed) is 0.000 < 0.05. It indicates a significant difference at the 95% level of confidence that there is an influence between the concentration of Pb in water and sediment on the growth of *Gracilaria* sp. [30].

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Table 2. Concentration of Pb in Water and Sediment

Sample	Heavy metal concentration (Pb) and BCF value		
	C Water	*C Sediment*	BCF Value
$P1_0$	0.90	3.57	3.967
$P2_0$	0.56	3.40	6.071
$P1_{20}$	0.24	7.61	31.708
$P2_{20}$	0.21	5.35	25.476
$P1_{40}$	0.09	1.37	15.222
$P2_{40}$	0.12	1.45	12.083

Description:
$P1_0$ = Pond 1 first sampling (age 0 days)
$P2_0$ = Pond 2 first sampling (age 0 days)
$P1_{20}$ = Pond 1 second sampling (aged 20 days)
$P2_{20}$ = Pond 2 second sampling (aged 20 days)
$P1_{40}$ = Pond 1 third sampling (aged 40 days)
$P2_{40}$ = Pond 2 third sampling (aged 40 days)

Low concentration of Pb heavy metal in the water because of Pb is mostly found in the sediment, thus indicating that most of the Pb compounds in Pond 1 and Pond 2 are in the form of particles or deposits. Anthropogenic materials such as Pb heavy metals carried by the air from factories and population activities will settle on sediments [31].

In Water and Holdfast of Gracilaria sp.

Bioconcentration factor of Pb on the seaweed showed in Table 3. The first sampling has a low Pb BCF because this sampling was carried out where *Gracilaria* sp. has not spread on the pond. Accumulatively, the Pb BCF value is still low, supported by using the t- test, where significance value (t-tailed) is 0.000 < 0.05. The results prove that the Pb heavy metal concentration varies significantly with the absorption of Pb heavy metal in the base of water [31].

Table 3. Heavy Metal Concentration (Pb) in Water and Holdfast of *Gracilaria* sp.

Sample	Pb concentration and BCF value		
	Water	Holdfast	BCF Value
$P1_0$	0.90	3.38	3.756
$P2_0$	0.56	2.75	4.911
$P1_{20}$	0.24	2.90	12.083
$P2_{20}$	0.21	3.66	17.429
$P1_{40}$	0.09	0.84	9.333
$P2_{40}$	0.12	0.81	6.750

Description:
$P1_0$ = Pond 1 first sampling (age 0 days)
$P2_0$ = Pond 2 first sampling (age 0 days)
$P1_{20}$ = Pond 1 second sampling (aged 20 days)
$P2_{20}$ = Pond 2 second sampling (aged 20 days)
$P1_{40}$ = Pond 1 third sampling (aged 40 days)
$P2_{40}$ = Pond 2 third sampling (aged 40 days)

In Water and Young Thallus of Gracilaria sp.

Pb BCF value at the young thallus of *Gracilaria* sp. showed in Table 4. Pb BCF value in water and the young thallus of *Gracilaria* sp. ranges from 4.5-10.208 (Table 4). By using the t-test, where significance value (t-tailed) is 0.000 < 0.05, so the hypothesis is proven that there is a difference absorption process in the tip of *Gracilaria* sp. for Pb heavy metals. The age of seaweed also greatly affects the process of Pb heavy metals sequestration in *Gracilaria* sp. [32].

Table 4. Heavy Metal Concentration (Pb) in Water and Young Thallus of *Gracilaria* sp.

Sample	Pb concentration and BCF value		
	Water	Young Thallus	BCF value
$P1_0$	0.90	4.24	4.711
$P2_0$	0.56	4.28	7.643
$P1_{20}$	0.24	2.45	10.208
$P2_{20}$	0.21	2.12	10.095
$P1_{40}$	0.09	0.85	9.444
$P2_{40}$	0.12	0.54	4.500

Description:
$P1_0$ = Pond 1 first sampling (age 0 days)
$P2_0$ = Pond 2 first sampling (age 0 days)
$P1_{20}$ = Pond 1 second sampling (aged 20 days)
$P2_{20}$ = Pond 2 second sampling (aged 20 days)
$P1_{40}$ = Pond 1 third sampling (aged 40 days)
$P2_{40}$ = Pond 2 third sampling (aged 40 days)

The results of bioconcentration factors indicate that *Gracilaria* sp. both at the holdfast and thallus can accumulate Pb heavy metals in the waters. A portion of heavy metals absorbed in water and accumulates in the form of particles and then settles on the surface of the sediment [33]. Metals will be bioaccumulated in living organisms or the body of aquatic biota. The amount of metal that accumulates will continue to increase [34].

Translocation Factors (*TF*)

TF analysis is used to calculate the Pb heavy metal translocation process from the base (holdfast) to the tip (young thallus) of *Gracilaria* sp. The value from the calculation of the Transaction Factor (TF), can be seen in Table 5 below.

TF value of Pb from holdfast to thallus is 1,015 so that *Gracilaria* sp. can be regarded as a high-value bioaccumulator. It was also checked with analysis to verify the existence of significant by using the t- test, where significance value (t-tailed) is 0.000 < 0.05. There are several factors that affect the difference in growth in holdfast and thallus *Gracilaria* sp. one of them is existance of heavy metal [33]. The concentration of Pb heavy metals contained in the holdfast and thallus of seaweed *Gracilaria* sp. and the absorption process influences the growth of

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Gracilaria sp. Value of TF > 1 shows that plants are enriched with metals as Pb heavy metal accumulators [35].

Tabel 5. TF Concentration on Holdfast to the Young Thallus of *Gracilaria* sp.

Sample	Pb concentration and TF value		
	Young Thallus	Holdfast	TF Value
$P1_0$	4.24	3.38	1.000
$P2_0$	4.28	2.75	2.000
$P1_{20}$	2.45	2.90	0.845
$P2_{20}$	2.12	3.66	0.579
$P1_{40}$	0.85	0.84	1.000
$P2_{40}$	0.54	0.81	0.667
Average			**1.015**

$P1_0$ = Pond 1 first sampling (age 0 days)
$P2_0$ = Pond 2 first sampling (age 0 days)
$P1_{20}$ = Pond 1 second sampling (aged 20 days)
$P2_{20}$ = Pond 2 second sampling (aged 20 days)
$P1_{40}$ = Pond 1 third sampling (aged 40 days)
$P2_{40}$ = Pond 2 third sampling (aged 40 days)

According to Bioconcentration factor (BCF) and Translocation Factors (TF), most of the investigations on microalgae and macrophytic algae were considered as valuable indicators because of their accumulation capacity [34]. Trace elements like manganese, copper, lead, and zinc are present in very small quantities and are considered as the essential micro-nutrients for proper growth of the plants. It is well known that elements such as Cu, Mo, Ni, Cl, and Zn are essential for plant growth in low concentrations [36]. Nevertheless, beyond certain threshold concentrations, these same elements become toxic for most plant species [37,38].

Several plant species are capable of tolerating high concentrations of heavy metals and thus opened new possibilities to use these plants to remediate contaminated soils (phytoremediation). Many studies have been conducted to determine the toxic levels of heavy metals for certain plants, especially those metals considered as public health threats [36,39]. At the low concentrations, some of the heavy metals excite some biological processes, but at threshold concentration these become toxic. Being non-biodegradable, these metals accumulate at various trophic levels through the food chain and can cause human health problems [40].

In humans, these metals hoard in living tissues and thus multiply the danger. Some metals cause physical distress while others may cause life-threatening illness, damage to the vital body system, or cause other damages. Thus, it is very essential to control the emission of heavy metals into the environment. Seaweeds are excellent agents of filtering the metals like zinc, cadmium, copper, nickel and iron and some potential carcinogens from seawater.

CONCLUSION

Highest Pb content was found in the second sampling, age 20 days, in Pond 1 for 7.61 ± 0.18 $mg.kg^{-1}$ and the Pond 2 for 5.35 ± 0.09 $mg.kg^{-1}$. This concentration has not exceeded the threshold value that might have an effect of more than 8.6 $mg.kg^{-1}$. Pb level analysis at the holdfast and thallus of *Gracilaria* sp. found the highest Pb content at age 0 days before planting, which is 3.38 ± 0.23 $mg.kg^{-1}$and decreases to post-harvest age of 40 days which is 0.84 ± 0.00 $mg.kg^{-1}$. Transaction Factor (TF) of heavy metal Pb value from holdfast to thallus is 1.015. so *Gracilaria* sp. absorbs heavy metals in high concentrations at the beginning of planting and is able to release it again before harvest time.

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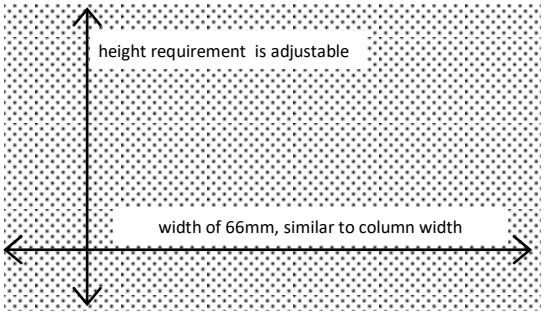

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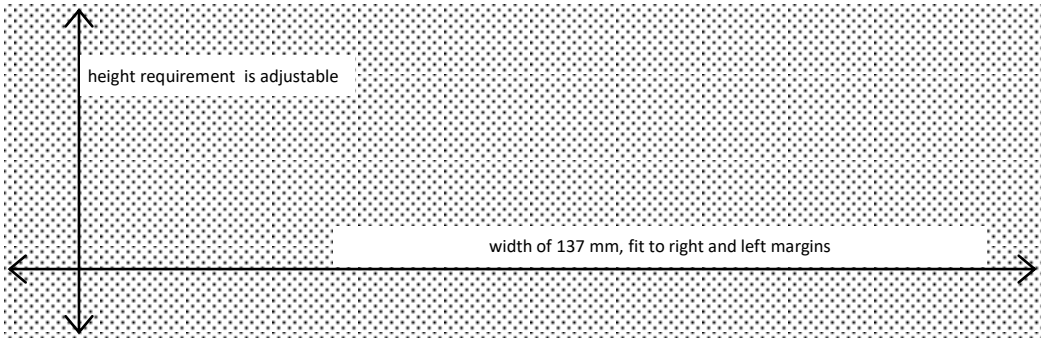

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Cover Images:

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

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