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A large graphic at the top of the page shows a molecular structure. It features a green and red ball-and-stick model of a complex organic molecule, possibly a protein or a large organic compound, set against a blue background with a grid of small dots. The molecule is partially enclosed by a red wireframe mesh. The overall shape of the graphic is a large, curved, triangular area pointing towards the top right.

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Isolation and Identification of Rhizosphere Bacteria Associated with Taro (*Colocasia esculenta* L. Schott) as Plant Growth Promoting Rhizobacteria

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Abstract

Taro is one of the food crops that has the potential to maintain food security. It has a high carbohydrate content and nutrients with a low glycemic index value. Taro is widely cultivated with intercropping techniques, especially in Kedungkandang District, Malang City. However, this technique caused a decrease in talas tubers production with only 7-10 tons ha⁻¹. This number was lower than the total optimal production with 20.7 tons ha⁻¹ of taro tubers. Plant Growth Promoting Rhizobacteria (PGPR), one of the most potential biofertilizers, can solve this problem with abilities such as phosphate-solubilizing, production of IAA, and nitrogen fixation. This research aimed to analyze the potency and identify the species of taro rhizosphere bacteria that had the best ability as PGPR agents. Taro rhizosphere bacteria were isolated using Pikovskaya medium and TSA (Tryptic Soy Agar) respectively. The PGPR abilities were evaluated on the phosphate-solubilizing, production of IAA, and nitrogen fixation. A total of 12 isolates for phosphate-solubilizing bacteria and ten isolates of IAA-producing bacteria. The highest concentration of phosphate solubilization was P1 isolate, with the concentration of 6.8 µg.mL⁻¹; while I4 isolate had the highest potency for IAA production with the concentration of 23.11 µg.mL⁻¹. Isolates P1 and I4 were selected for the nitrogen fixation ability test. P1 isolate shows the highest ammonia concentration of 2.52 µg.mL⁻¹, and it was identified as *Stenotrophomonas rhizophila* with a similarity of 99.91% with *Stenotrophomonas rhizophila* R2A2 67. The potential isolates can be used as PGPR agents or biofertilizers to increase the production of taro plants.

Keywords: *Colocasia esculenta*, IAA, Nitrogen, PGPR, Phosphate.

INTRODUCTION

In Indonesia, agricultural sector plays a crucial role to fulfill food consumption for national economic resilience [1]. Food production is required to support population growth, where in the last 20 years, Indonesia's population has increased by 63.9 million people, this number equivalent to 26.9% of the total population in 2010 [2]. The increasing number of the population every year also increases the country's food needs [3].

The government encourages national food security with several strategies such as increasing agricultural production, optimizing and expanding land, as well as developing food diversity other than rice [4]. The taro commodities are one of the potential food contributing to food security in terms of their production potential [5]. Taro is a highly nutritious source of carbohydrates with a lower glycemic index value of 54 compared to other tuber crops such as potatoes and rice.

Kedungkandang District, which is the location of the sampling area, generally uses the

intercropping technique, namely the technique of planting several types of plants in one area with a predetermined plant distance. The intercropping technique causes a decrease in plant growth due to limited nutrients and water, which results in a decrease in tuber productivity [6,7]. The optimal production potency of taro tubers can reach 20.7 tons ha⁻¹, but observations show that production only reaches about 7-10 tons ha⁻¹ [8].

Biofertilizer is a fertilizer that contains microorganisms and is practical for increasing soil fertility and crop production [9]. One of the biofertilizers that are often used and have the potency for agricultural resilience can help ensure the availability of potential nutrients for plants and increase the use of nutrients more efficiently, namely Plant Growth Promoting Rhizobacteria (PGPR) [10].

PGPR has abilities, such as phosphate solubilization, which is required in all major metabolic processes in plants, as well as energy transfer, signal transduction, photosynthesis, and respiration [11]. Another ability for nitrogen fixation, which is the most important nutritional element for plants after phosphate and the production of phytohormones IAA, is a hormone that is very influential on plant growth [12]. Based on research from Soubeih and El-Sayed

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[13] on potato and garlic plants, using an intercropping technique with biofertilizer agents, namely *Azotobacter chroococcum*, *Bacillus megaterium*, *Thiobacillus thioparus*, showed increased growth from potato sprouting to height and weight of plants, leaf width, also increased garlic and production potato tuber.

Combination treatment among biofertilizer agents and minerals showed significant results in the growth and yield of tubers and cormels on taro plants [14]. The use of biofertilizer agents such as *Azotobacter* with nitrogen fixation potential, Vesicular Arbuscular Mycorrhizae (VAM), and Phosphate solubilizing bacteria (PSB) can minimize the use of inorganic fertilizers as well as increase the harvest index and dry matter content of roots, corms and leave of taro plants [15]. Therefore, this research aims to analyze the potential and identify the species of taro rhizosphere bacteria that have the best ability as PGPR agents.

MATERIAL AND METHOD

Taro Rhizosphere Soil Sampling

Soil samples were obtained from taro agricultural land in Tlogowaru Village, Kedungkandang District, Malang, East Java, Indonesia at 582 m.a.s.l (08°01'38.4"S 112° 40' 16.3"E). Samples were taken compositely at one location from three sampling areas, each area consisting of three plants. The sample had taken at 3-5 points per plant in the soil around the root area. Soil samples were taken using plastic slips, then stored in a cool box and brought to the Microbiology Laboratory, Faculty of Mathematics and Natural Sciences, Brawijaya University. Physicochemical measurements include the C/N ratio, pH, organic matter, and water content. The C/N ratio and organic matter were analyzed in the Soil Laboratory of the Faculty of Agriculture, Brawijaya University.

Isolation of Taro Rhizosphere Bacteria

A total of 25 g of soil sample was diluted into 225 mL of 0.85% NaCl solution [16]. The serial dilution was made from 10^{-1} – 10^{-6} . A total of 0.1 mL aliquot of the sample suspension was poured into a Petri dish containing Pikovskaya agar medium for isolation of phosphate solubilizing bacteria and TSA medium for isolation of IAA producing bacteria, respectively. The Pikovskaya agar consists of (g.L⁻¹) Glucose 5, CaHPO₄ 2.5, KCl 0.1, (NH₄)₂SO₄ 0.25, NaCl 0.1, MgSO₄.7H₂O 0.025, MnSO₄.H₂O 0.25, FeSO₄.7H₂O 0.25, yeast extract 0.25 and agar 10. The TSA medium consisted of TSA 40 g.L⁻¹, L-tryptophan 200 µg.mL⁻¹. The

culture was incubated at 30°C for 48-72 hours. Each isolates that grown on TSA and Pikovskaya agar media was observed and enumerated based on the Total Plate Count (TPC) formula and was purified using the spread plate method.

Quantitative Assay of Phosphate Solubilizing Bacteria

The ability of bacteria to solubilize phosphate was carried out by a quantitative test based on Ahmad *et al.*, [17] with minor modification. A total of one loop of bacterial culture were inoculated into 15 mL of Pikovskaya broth medium (pH 7) and then incubated in a shaker at 120 rpm, at 30°C, for 48 hours, then the OD was equalized. A total of 2 mL cultures were inoculated into 20 mL Pikovskaya broth medium (pH 7) then incubated in a shaker at 120 rpm, temperature of 30°C for 72 hours. Then 2 mL of culture were centrifugated at 10.000 rpm for 20 minutes, cultures were taken at 0, 24, 48, 72 hours. A total of 1 mL supernatant was homogenized with 10 mL of Molybdate reagent and 40 mL of distilled water, then added 0.1 mL of SnCl₂. The suspension was incubated for 10 minutes at 30°C until the color changed to blue. The absorbance value was measured using a spectrophotometer at 660 nm. The concentration of phosphate solubilizing concentration was calculated.

Quantitative Assay of IAA-Producing Bacteria

The ability of bacteria to produce IAA was carried out using a quantitative test [18]. A total of one loop of bacterial culture was inoculated into 25 mL of Tryptic Soy Broth (TSB) medium with 2% L-Tryptophan added, then incubated in a shaker at 120 rpm at 30°C for 48 hours and then the OD was equalized. Four milliliters of cultures were inoculated in 40 mL TSB with 2% L-Tryptophan and incubated in a 120 rpm shaker at 30°C for 72 hours. Three milliliters of culture were centrifuged at 0, 24, 48, and 72 hours at 10,000 rpm for 10 minutes. Two-milliliter supernatant was homogenized with 4 mL of Salkowsky's reagent. The suspension was incubated in a dark room for 30 minutes or until the suspension color turns pink. The absorbance value was measured using a spectrophotometer at 530 nm.

Quantitative Assay of Nitrogen Fixation Bacteria

Isolates that showed the best ability in the previous test were used for the nitrogen fixation test. One loop of culture was inoculated on semisolid N-free media with the addition of

bromothymol blue and incubated at 30°C for 72 hours. Isolates in the medium that changed color from green to bluish were used for quantitative testing based on Setia *et al.* [19] with minor modification. One loop of bacterial culture was inoculated into 10 mL of N-free (without Bromothymol Blue) and incubated in a shaker at 120 rpm at 30°C for 24 hours. Ten milliliters of culture were inoculated into 90 mL of N-free medium and incubated in a 120 rpm shaker at 30°C for 72 hours. Fifteen milliliters of culture at 0, 24, 48, and 72 hours incubation were centrifuged at 10.000 rpm for 10 minutes. Ten milliliters of the supernatant were added with the sera ammonia test kit, homogenized, and incubated for 5 minutes at room temperature 28-30°C, and the color changed to green. The absorbance of suspension was measured using a spectrophotometer at 700 nm. Ammonia concentration values were calculated based on the standard ammonia curve.

Identification of Bacteria Based on 16S rDNA

Potential PGPR bacterial chromosomal DNA was extracted using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (ZYMO RESEARCH, USA). Amplification of 16S rDNA sequences using universal primers 27f (5'-GAG AGT TTG CTG GCT ATC CAG-3') and 1492r (5'-CTA CGG CTA TGT CCT TAC GA-3'). The composition of the PCR mix was 94°C (5 minutes) followed by denaturation (94°C; 0.5 minutes), annealing (55°C; 0.5 minutes), extension (72°C; 1.5 minutes) to 35 cycles and post-extension (72°C, 7 min) [19]. The 16S rDNA amplicon was sequenced at First Base Malaysia. The 16S rDNA sequences were aligned with reference strains from the GenBank database. Construction of phylogeny tree using MEGA 11 program, according to Neighbor-Joining algorithm with 1000 bootstraps.

Data analysis

The quantitative data of phosphate-solubilizing, IAA production, and nitrogen-fixing bacteria was analyzed based on a Two-Way ANOVA. It was followed by a Tukey test using the SPSS 26 programs trial edition.

RESULT AND DISCUSSION

Soil Physicochemical Environmental Parameter Analysis

The results of soil environmental parameters (Table 1) were assessed based on criteria that referred to data from the Soil Research Center (Balai Penelitian Tanah) [20]. Soil pH can indicate

nutrient content in the soil and can have an impact on the existence of microorganisms in the soil [21]. The pH of the soil was categorized as neutral, while the C-organic and N-total content were in a low category, and the organic matter content was classified on average. From these results, it can conclude that the acidity of the soil is not optimal for taro growth. According to Setiawan *et al.* [22], soil with low pH or acidic has an impact on stunted plant growth. But this can be solved by providing phosphate solubilizing bacteria that can produce organic acids, so it can increase soil alkalinity and provide dissolved phosphate for plant growth [23].

Table 1. Soil physicochemical and environmental parameters

No.	Parameters	Value
1	Light intensity (×100 lux)	132.000
2	Humidity (%)	73.000
3	Soil temperature	27.100
4	C - organic matter (%)	1.135
5	N total (%)	0.116
6	C/N Ratio	9.330
7	Organic matter (%)	1.920
8	Moisture content (%)	56.150
9	Soil pH	7.530
10	Density of phosphate-solubilizing bacteria (10 ⁵ CFU.g ⁻¹)	36.800
11	Density of bacteria producing IAA (10 ⁵ CFU.g ⁻¹)	121.260

Carbon is an important element that indicates soil fertility, plays a major role as a food source for microorganisms, and can affect the abundance of phosphate and nitrogen. The elements decomposed by microorganisms. C-organic and N-total are categorized as less than optimal to support plant growth. The low carbon element in the soil causes a lack of food sources for microorganisms resulting in less decomposition of nitrogen elements [24]. The content of organic matter is categorized as average so that it's able to support plant growth. Organic matter has an impact on increasing the abundance of microorganisms and the growth of plant roots [25].

Quantitative Test of Plant-growth Promoting Potential

Phosphate Solubilizing Bacteria

A total of 12 isolates with different morphology showed different phosphate concentrations at each incubation time (Fig. 1). P1 isolate has the highest phosphate solubilizing potency with a concentration of 6.8 µg.mL⁻¹ at 48 hours, compared to other isolates. The isolates

such as P1, P8, P9, P12 also showed the highest concentration at 48 hours. The other isolate with the highest concentration was P12 with a concentration of 5.43 $\mu\text{g.mL}^{-1}$ at 48 hours, followed by P8 at 5.41 $\mu\text{g.mL}^{-1}$ at 48 hours. In this study, P1 which was identified as *Stenotrophomonas rhizophila* had a higher phosphate solubilization concentration 6.8 $\mu\text{g.mL}^{-1}$ than *Stenotrophomonas maltophilia* IMP289 with a concentration of 0.13 $\mu\text{g.mL}^{-1}$ and *Stenotrophomonas maltophilia* 46 0.43 $\mu\text{g.mL}^{-1}$ and *Stenotrophomonas maltophilia* $\mu\text{g.mL}^{-1}$ [26]. Variations results in phosphate concentrations from bacteria are influenced by different types of microorganisms caused by the influence of pH, humidity, and soil temperature so that the population and diversity of microorganisms are also more diverse [12].

IAA Production Bacteria

A total of 10 isolates with different morphology showed different IAA production abilities (Figure 2). Isolate I4 had the highest IAA production with a concentration of 23.11 $\mu\text{g.mL}^{-1}$ at 48 hours and 14.43 $\mu\text{g.mL}^{-1}$ at 72 hours, while isolates I7 had the highest IAA concentration of 10.06 $\mu\text{g.mL}^{-1}$ at 48 hours and 8.24 $\mu\text{g.mL}^{-1}$ at 72 hours. A decrease in the IAA concentration of isolates I4 and I7 occurred at 72 hours of incubation, then followed by isolate I9 with 8.87 $\mu\text{g.mL}^{-1}$ concentration at 24 hours. The decrease in concentration could be due to the bacteria having passed the optimal phase and the lack of nutrients in the medium, so the bacteria remodel the hormones produced to be used in the growth process [27].

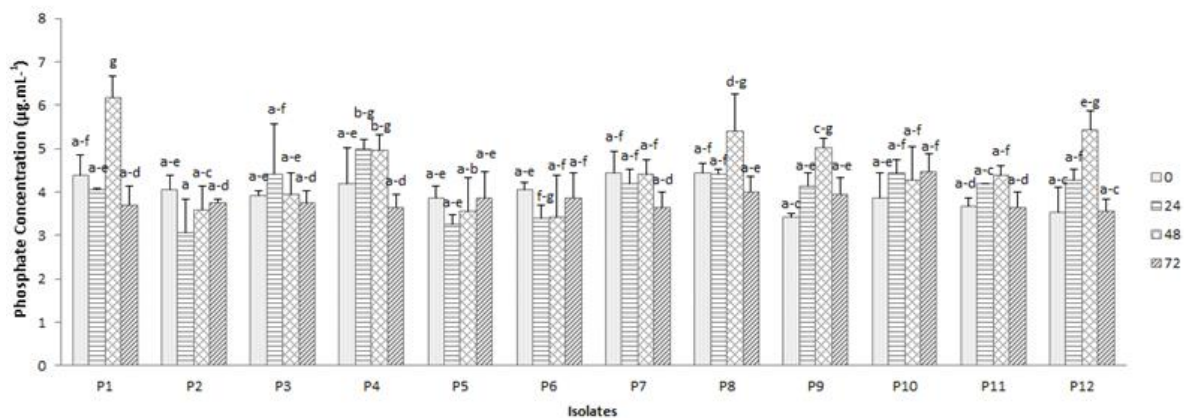


Figure 1. The concentration of dissolved phosphate by bacteria at different incubation times
*Data were analyzed using two way ANOVA at $\alpha=0.05$. It describe the difference in phosphate-solubilizing among isolates and incubation time ($p<0.05$).

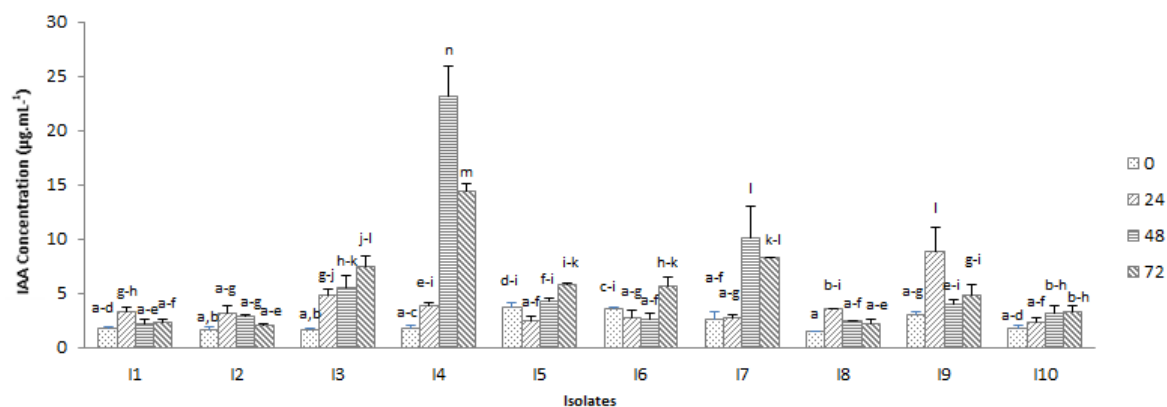


Figure 2. The concentration of IAA hormone production by bacteria at different incubation times
*Data were analyzed using two way ANOVA $\alpha=0.05$. It describe the difference in IAA production among isolates and incubation time ($p<0.05$).

This result was higher than some plant rhizosphere bacteria such as isolate S7.3 with a

concentration of 7.944 $\mu\text{g.mL}^{-1}$ in suren plants [28] and star fruit isolates at 16.71 $\mu\text{g.mL}^{-1}$ [29]

much higher than isolates from corn plant-bacteria with a concentration of $0.9361 \mu\text{g.mL}^{-1}$ [30]. The addition of L-Tryptophan to liquid media according to Mohite [31] is considered as IAA precursor because it can increase the production of IAA in culture, media with the addition of L-Tryptophan has a higher production than that which is not added. Some examples of potential IAA-producing bacteria such as *Pseudomonas* sp., *Bacillus* sp., *Azotobacter* sp. Several strains showed different concentrations of production, and even strains in the genus *Bacillus* produced different IAA concentrations. IAA production is influenced by culture conditions, growth phases, and substrate availability [32].

Nitrogen Fixation Bacteria

Bacterial isolates that had the highest ability in phosphate solubilizing and IAA production were selected for the nitrogen fixation test. I4 and P1 isolates showed a blue color change on Nitrogen Free Bromothymol Blue (Nfb) medium. Thus, both isolates were selected for the quantitative test of nitrogen fixation ability.

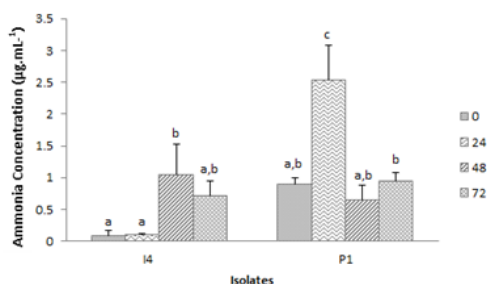


Figure 3. Ammonia concentration by bacteria at variant incubation times

*Data were analyzed using two way ANOVA at $\alpha = 0.05$. It describe the difference in ammonia production among isolates and incubation time ($p < 0.05$).

Isolate P1 with the highest concentration of $2.52 \mu\text{g.mL}^{-1}$ at 24 hours and decreased at 48 hours to $0.65 \mu\text{g.mL}^{-1}$. Isolate I4 with the highest concentration of $1.04 \mu\text{g.mL}^{-1}$ at 48 hours and decreased to $0.72 \mu\text{g.mL}^{-1}$ at 72 hours of incubation. From these results, it was concluded that isolate P1 had a higher nitrogen fixation ability than isolate I4 (Fig. 3). The comparison of the ability to produce ammonia will be different for each type of microorganisms and can be caused by the variation of bacteria type, oxygen content in the growth medium, as well as the variation in carbon sources that can affect the ability of bacterial cells to excrete ammonium [33].

Phylogenetic tree of Rhizosphere Bacterial Species Based on 16S rDNA

Three isolates with the best ability were selected in each test, isolates P1, P8, P12 with the ability of phosphate-solubilizing and isolates I4, I7, I9 with the ability of IAA producing. The six isolates were tested for pathogenicity using blood agar media. Isolate P1 and I7 did not show lysis on blood agar media, so the isolate was concluded as non-pathogenic. Isolate P1 was chosen as the potential PGPR isolate, with the ability to solubilize phosphate and nitrogen fixation, was constructed using a phylogenetic tree based on 16S rDNA and compared with the reference strain. The P1 was identified as *Stenotrophomonas* at 99.91% similarity with *S. rhizophila* R2A2 67 (Fig. 4). *Stenotrophomonas rhizophila* is a rhizosphere bacteria and plant endosphere bacteria in all plant phylogenies. These bacteria produce osmoprotective substances and provide protection from pathogens, either bacteria or fungi [26].

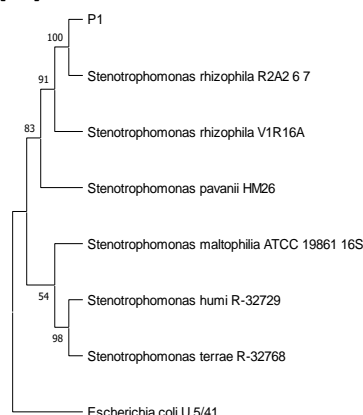


Figure 4. Phylogeny tree of potential PGPR isolates P1 and reference strains according Neighbor-Joining algorithm with 1000 bootstraps, using MEGA 11 program

The *S. rhizophila* was rarely studied for PGPR, especially for Phosphate-Solubilizing Bacteria (PSB) and Nitrogen Fixing Bacteria (NFB). However, the type of *S. maltophilia* has been confirmed to have the presence of the *nifH* gene [34]. According to Kumar and Audipudi [35], *S. maltophilia* AVP27 from chili plant rhizosphere has the ability to produce $80 \mu\text{g.mL}^{-1}$ of ammonia and confirm to dissolve phosphate [36]. However, *S. maltophilia* has pathogenic characteristics caused by its lower optimum growth temperature [37] than *S. rhizophila*.

CONCLUSIONS

P1 isolates from the rhizosphere of the taro plant in Tlogowaru Village showed the highest potential for phosphate solubilizing with the concentration of $6.8 \mu\text{g.mL}^{-1}$ at 48 hours and nitrogen fixation of $2.52 \mu\text{g.mL}^{-1}$ at 24 hours. P1 isolate was identified as *Stenotrophomonas rhizophila* with similarity (99.91%). P1 isolate has the ability to be used as a biofertilizer agent for the cultivation of taro plants.

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***Phaleria macrocarpa* Leaves Extract Reduce Tumors Growth and Improve Histological Changes of Liver and Kidney on 4T1 Breast Cancer Mice Model**

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Abstract

Breast cancer is a type of cancer that highly occurs globally and causes death cases. Of the many ways of treating breast cancer, chemotherapy is the most recommended, even though it causes various disturbing side effects. Therefore, alternative medicine using bioactive compounds of medicinal plants has begun to be widely used, for example, *Phaleria macrocarpa*, a plant native to Indonesia with anticancer and antioxidant activity. The liver and kidneys are important organs that function to maintain body homeostasis. The use of crude extracts of medicinal plants often causes damage to those organs at inappropriate doses. This research aimed to get an effective dose for reducing breast cancer growth and is safe for the liver and kidneys. A total of 36 mice were divided into six groups, including healthy control, cancer control, cisplatin, and three doses of *P. macrocarpa* extract (58.9, 117.8, and 235.6 mg.kg⁻¹). The experimental animals were injected using a 4T1 cell line and treated orally using *P. macrocarpa* leaf ethanol extract for two and three weeks. The tumor volume of mice was measured periodically. At the end of treatment, mice were sacrificed, and their liver and kidney organs were isolated. Both organs were then prepared for H&E staining and observed using a microscope. The results showed that a dose of 58.9 mg.kg⁻¹ and 117.8 mg.kg⁻¹ of *P. macrocarpa* extract could reduce tumor volume by more than 90%, and the 117.8 mg.kg⁻¹ dose is the safest dose to use because it does not affect the kidney and cause chronic damage to liver tissue.

Keywords: extract, kidney, liver, tissue damage, tumor.

INTRODUCTION

Worldwide, breast cancer comprises 10.4% of all cancer incidences among women, making it the fifth most common cause of cancer death. Breast cancer refers to the abnormal growth and proliferation of cells originating in breast tissue due to disturbed and unregulated cell cycles [1]. Indonesia alone has more than 300,000 new breast cancer cases in 2020, most of them in women [2]. This problem needs to be solved because it metastasizes into several organs such as lungs, bones, and liver [3]. The efforts to treat and cure breast cancer depend on tumor grade, hormone receptor status, metastatic potential, patient profile, and many other things. Several treatments can be used to treat breast cancer, including surgery, radiation therapy, and chemotherapy. Chemotherapy is the most recommended treatment, but like other therapeutic agents, chemical drugs for chemotherapy can be toxic to normal tissue and have fairly obvious side effects. Up to 80% of the patients have a risk to went through vasomotor syndrome [4] and other symptoms, including

nausea, vomiting, diarrhea, fatigue, hair loss, and psychological stress [5].

Indonesia is a country that has the second-largest biodiversity in the world and has many potential plants used in traditional medicine but has no scientific evidence [6]. *Phaleria macrocarpa* is an original plant from Indonesia, precisely in the tropical region of Papua. This plant has been widely reported to have medical activities such as anti-tumor, anti-hyperglycemic, anti-inflammatory, antioxidant, and anti-microbial. The leaves of *P. macrocarpa* have been used to treat various types of cancer, including breast cancer. The methanol extract from *P. macrocarpa* can work as an anti-proliferative, anti-angiogenic, and apoptotic inducer due to its main compounds, phalerin, and gallic acid [7]. Based on in vitro research conducted by Christina *et al.* [8], ethanol extract of *P. macrocarpa* leaves demonstrated cytotoxic activity of 50% at a dose of 97 µg mL⁻¹ in a breast cancer cell line. In addition, the high content of phenolic and flavonoid compounds makes this extract can be used as a natural anticancer and antioxidant to replace or complement modern treatment methods.

The high interest in using herbal medicine as an alternative drug demands a scientific evaluation to assess the toxicity of these medicinal drugs. Using crude extract containing

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various phytochemicals is feared to have side effects if used at inappropriate doses [9]. The liver is a crucial organ of the human body that detoxifies various xenobiotics such as drug metabolites and helps maintain homeostasis. Liver cells frequently experience stress during this detoxification process due to oxidative damage from free radicals [10]. However, the kidney maintains metabolism and homeostasis in the body as well. The kidneys have a crucial role in excretion by forming urine by filtering harmful or excessive substances for the body [11].

Because of those functions, the kidney and liver are target organs often used to observe the effect or toxicity of drugs or other chemical substances. Histopathology is the most appropriate screen evidence for the kidney and liver because this method is convenient enough to detect diseases that occur in a short time in experimental animals in the laboratory [12]. *Phaleria macrocarpa* anticancer effect has been proven for breast cancer cells, but it still has limited study to confirm the effect on the liver and kidneys. Thus, this research aimed to examine the impact of various *P. macrocarpa* leaf extract doses to find an effective dose to treat breast cancer while not having side effects on other vital organs.

MATERIAL AND METHODS

Plant Material and Extraction

The leaves of the *P. macrocarpa* plant were dried and grounded to a powder form. The powder was then macerated with 70% ethanol for 24 hours while stirring several times. The ethanol extract obtained concentrated using a vacuum pump evaporator at a low pressure of 50°C until it forms a paste. The extract will be dissolved in water and given for 28 days through oral injection.

Cell Line

4T1 cell line derived from mice breast cancer and a type of TNBC was obtained from Cancer Chemoprevention Research Center (CCRC), Faculty of Pharmacy, University of Gadjah Mada, Yogyakarta. Cells were then cultured on DMEM complete media (DMEM, 10% FBS, and 1% penicillin-streptomycin) and incubated at 37°C with 5% CO₂ content. The culture cells will be split 2-3 times a week to reach confluency (60%-80%).

Breast Cancer Induction

The experimental animal (*Mus musculus*) was obtained from the Laboratory of Animal Physiology, Department of Biology, State Islamic

University of Malang, aged 5-6 weeks old. The animal research protocol has been reviewed and approved by Animal Care and Use Committee, Brawijaya University, Indonesia (Approval number 025-KEP-UB-2021).

Induction of breast cancer was done by 4T1 cell injection dissolved in PBS at 100µl. 4T1 cell lines (3×10⁶ cell.mL⁻¹) was injected into the subcutaneous part of the mammary glands of mice three times in two weeks (4-5 days interval into the next injection). Then we observed whether they experienced toxic symptoms such as weight loss, changes in appetite, or other clinical signs in the body. It is our modification of Pulaski [13]. The bulge that appears at the injection point was observed, and if the tumor bulge has reached a volume of 100-300 mm³, mice are ready to be given further treatment [14].

Experimental Design

A total of 18 female BALB/C mice were divided into six groups with three animals each. The mice were acclimatized for seven days before being given treatment. Mice were then divided into several treatment groups, as follows:

- Control - : Mice were not induced by breast cancer and were not given the extract.
- Control + : Mice induced by breast cancer and not given the drug/extract.
- Cisplatin : Mice induced by breast cancer and injected intraperitoneally with 4 mg.kgBW⁻¹ of cisplatin [15].
- Dose 1 : Mice induced by breast cancer and treated with 58.9 mg.kg⁻¹ of *P. macrocarpa* leaves extract.
- Dose 2 : Mice induced by breast cancer and treated with 117.8 mg.kg⁻¹ of *P. macrocarpa* leaves extract
- Dose 3 : Mice induced by breast cancer and treated with 235.6 mg.kg⁻¹ of *P. macrocarpa* leaves extract.

Tumor Volume Measurement

Tumor length (L) and width (W) were measured three times per week using a calliper. The data is then calculated using a formula based on the Faustino-Rocha [16] reference:

$$V = \frac{(W^2 \times L)}{2}$$

Description:

- V = Tumor Volume
- W = Tumor width
- L = Tumor length

Histopathology Analysis

After the treatment, mice were sacrificed by dislocation to isolate several organs, including breast, liver, and kidney, for histopathology analysis. The organs obtained were then immersed in a fixative solution (10% formalin) and prepared using paraffin wax. The preparations were cut with a thickness of 5-7 mm and then stained using hematoxylin and eosin (H&E) staining. After that, the samples were observed using an Olympus BX51 microscope and photographed using OptiLab 3.0 software.

RESULT AND DISCUSSION

Breast Tumor Volume

Figure 1 demonstrated a decrease in tumor volume after treatment with *Phaleria macrocarpa* ethanol extract. The reduction in tumor volume until the third week in the control treatment occurred by 77%, while treatment with cisplatin decreased by 98%. However, the tumor volume for doses 1, 2, and 3 seems to be reduced by 100%, 94%, and 87% in order. A 58.9 mg.kg⁻¹ *P. macrocarpa* was the most effective dose to treat breast cancer in vivo. The active compounds of *P. macrocarpa* leaves extract effectively inhibit the growth of cancer cells, characterized by a decrease in tumor volume. Based on the in silico study, *P. macrocarpa* extract has bioactive compounds such as the lignan group that stimulate Caspase 3 and Bax proteins. In addition, the flavonoid group can also act as a Bcl-2 inhibitor so that it can be used as an apoptotic agent [8].

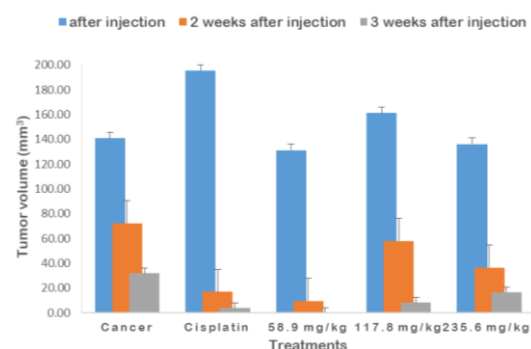


Figure 1. The decrease of breast tumor volume in all groups after two weeks and three weeks of treatment.

The results showed that *P. macrocarpa* extract groups worked as effectively as the cisplatin groups. Cisplatin or cis-diamminedichloroplatinum (CDDP) is a chemotherapy drug used to treat various types of cancer. Cisplatin compound consists of two chloro and two ammine ligands. This complex

compound reacts in vivo, binds, and causes DNA crosslinks, triggering apoptosis [17]. Cisplatin could induce apoptosis and trigger cell death by DNA damage through many pathways, including reactive oxygen species and binding to the N7 reactive center on purine residues [18].

Several bioactive compounds are found in *P. macrocarpa*, and gallic acid is the most widely isolated and studied natural antioxidant [19]. Gallic acid is a polyhydroxy phenolic compound that can be found in various natural ingredients in nature. Various studies showed that it has an anticancer activity that has been tested in vitro and in vivo by inhibiting cell proliferation and inducing apoptosis [19,20]. Gallic acid can cause cell cycle perturbation in the G1 phase and interfere with the mitotic phase in cancer cells. Another study suggested that gallic acid induces DNA fragmentation through caspase activation and cell cycle arrest via decreased Cdks and cyclin protein levels [20].

Histology of Liver

The liver and renal organs was observed to determine the effect of *P. macrocarpa* on other crucial organs. Liver histology of control mice that were not exposed to the *P. macrocarpa* extract appeared mainly in normal structure (Fig. 2). The hepatic cells have normal nuclei and are arranged toward the central vein. Meanwhile, cisplatin and *P. macrocarpa* extract treatment can change the normal structure and cause abnormalities characteristics. Figure 3 showed the presence of sinusoidal dilatation, blood vessel dilatation, and congestion in cisplatin treatment [21]. Cisplatin has several toxicities and side effects, including hepatotoxicity and nephrotoxicity. Because cisplatin can accumulate in the liver and kidney cells, enhancing the production of reactive oxygen species [17]. Cisplatin induces liver damage such as cytoplasmic changes, especially in cells around the central vein. A higher dose of cisplatin can cause hepatocellular vacuolization and sinusoidal dilatation [22].

The histopathology changes on three doses of *P. macrocarpa* extract (Fig.4). Figure 4 represents treatment with dose 1 of *P. macrocarpa* extract, showing various liver abnormalities. The most visible part is the hydropic degeneration which makes the hepatocyte structure looks not solid. Hydropic degeneration is cellular swelling, an acute reversible hepatocyte change. Cells with hydropic degeneration will look enlarged, with clear cytoplasm but with a normal nucleus [23].

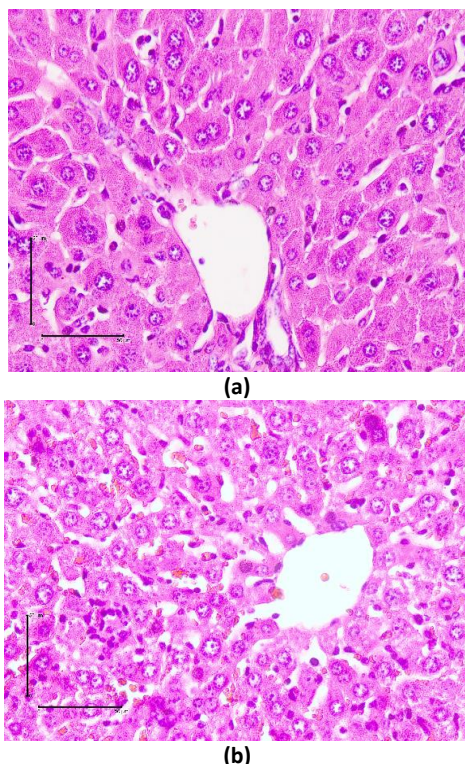


Figure 2. Normal liver histology on: control (a) and cancer (b) treatment at 400x magnification. The bar at the bottom left represent 50 µm.

These swollen cells can end up releasing the cell content into the ECM and causing necrotic cell death. Therefore, the area with this kind of cell injury is also likely to show a necrotic area [24]. Hydropic degeneration increases intracellular water by ion and fluid homeostasis [25]. It also indicates mild dilatation and congestion of the existing central vein. In addition, infiltration of inflammatory cells was also found at several points.

Figure 5 is a representative picture of treatment with dose 2 of *P. macrocarpa* extract. The structure of hepatocytes in the second dose did not appear to have a severe hydropic

degeneration as in the first dose. Although infiltration of inflammatory cells at some points and dilatation in the central veins and blood vessels, the congested blood vessels were not as many as the previous dose. Figure 6 is a representative picture of treatment with dose 3 of *P. macrocarpa* extract. Observations at 100x magnification showed less organ damage than the previous doses. There was mild dilatation in the portal vein and cloudy swelling surrounded by hydropic degeneration, also categorized as mild. There were no infiltration of inflammatory cells and no Kupffer cells activation seen in the picture. These results agree with the research conducted by Sundari *et al.* [26] that *P. macrocarpa* extract has hepatoprotective activity because it reduces the level of liver tissue damage, including necrosis and degeneration of liver cells. Flavonoids in *P. macrocarpa* act as antioxidants that eliminate free radicals by releasing the hydrogen atoms from their hydroxyl groups. The hydroxyl group of flavonoids can accommodate superoxide radicals and prevent the damage of membrane lipids that damage tissues [27].

Damaged cell membranes and proteins result from oxidative stress caused by free radicals. Oxidative stress is a condition in which there is an imbalance between reactive oxygen species (ROS) such as hydrogen peroxide and antioxidative compounds such as SOD. ROS, such as hydrogen peroxide, superoxide, hydroxyl radical, etc., can cause oxidative damage such as lipid peroxidation, amino acid oxidation, cross-links protein formation, to DNA strands ruptured. However, SOD is an enzyme that is very important in converting superoxide into H_2O_2 to prevent damage at the cellular level because H_2O_2 is less reactive [28].

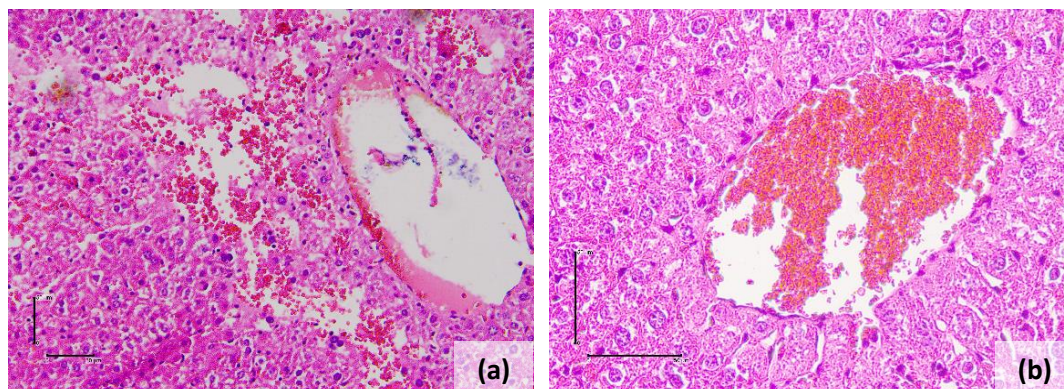


Figure 3. Liver histology on cisplatin treatment at 200x (a) and 400x magnification (b). The bar at the bottom left represents 50 µm.

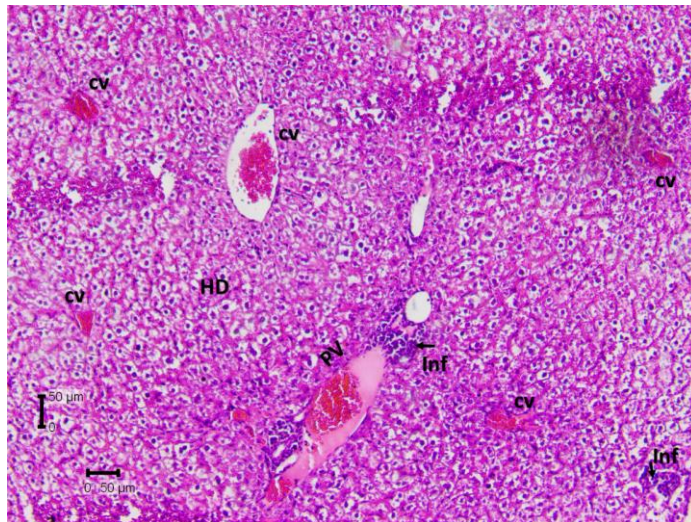


Figure 4. Liver histology on dose 1 of *Phaleria macrocarpa* extract treatment at 100× magnification. The bar at the bottom left represent 50 μm. (Notes: cv= central vein, pv= portal vein, hd= hydropic degeneration, inf= inflammation).

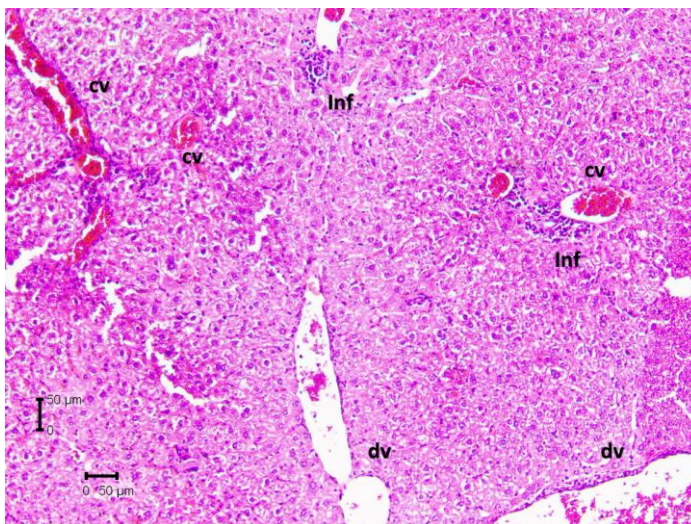


Figure 5. Liver histology on dose 2 of *Phaleria macrocarpa* extract treatment at 100× magnification. The bar at the bottom left represent 50 μm. (Notes: cv= central vein, dv= dilated vessel, inf= inflammation).

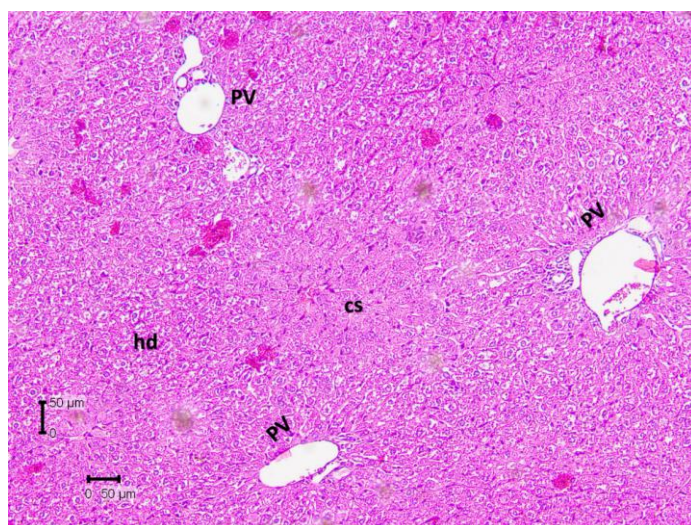


Figure 6. Liver histology on dose 4 of *Phaleria macrocarpa* extract treatment at 100× magnification. The bar at the bottom left represent 50 μm. (Notes: cs= cellular swelling, hd= hydropic degeneration, pv= portal vein).

This study showed that *P. macrocarpa* extract has a side effect on liver tissue and hepatoprotective activity at a higher dose. The fruit and leaves of *Phaleria macrocarpa* contain flavonoids and phenolics, which are antioxidant agents. The antioxidant activity of this extract is associated with free radical scavenging activity [7] and induces the production of superoxide dismutase [29]. Protective actions against ROS are performed by several enzymes, including superoxide dismutase (SOD), catalase and glutathione peroxidase. A nonenzymatic compound, such as tocopherol, vitamin E, beta-carotene, and ascorbate, has the same function [30].

Histology of Renal

The next results are the gross examination of renal histology. Figure 7 shows the kidney histological observations on healthy control and cancer without any significant differences. Both control treatments showed a fairly large area without any major damage to either the tubules or the glomerulus. There was only a small amount of bleeding in the dilated blood vessels and mild inflammatory cells. Inflammation is the body responds to external and internal stimuli. Inflammation caused by tissue damage can be characterized by increased blood flow and vascular permeability, accompanied by the accumulation of leukocytes and other inflammatory mediators such as cytokines [31]. Figure 8 showed vascular bleeding in the glomerulus and between the tubules in the cisplatin treatment. Mild tubular necrosis, especially in proximal tubules marked by pyknotic nuclei also shown in the picture. The accumulation in kidney tissue cells is the basis for cisplatin-induced nephrotoxicity.

Oxidative stress was implicated in kidney injury and liver injury by cisplatin. These histological changes after cisplatin treatment confirm irreversible kidney injury, which develops from inflammation and oxidative stress that cause vascular damage. Cisplatin causes the injury by interfering with mitochondrial function and maintaining calcium homeostasis [32]. Cisplatin injures multiple renal compartments, including blood vessels, glomeruli, and tubules. Chloride on cisplatin is one of the molecules that promote kidney injury. The chloride goes through the cell, increases intracellular concentrations, and triggers intracellular injury pathways, including caspase activation, cyclin-dependent

kinases, mitogen-activated protein kinase activation, and p53 signaling [33].

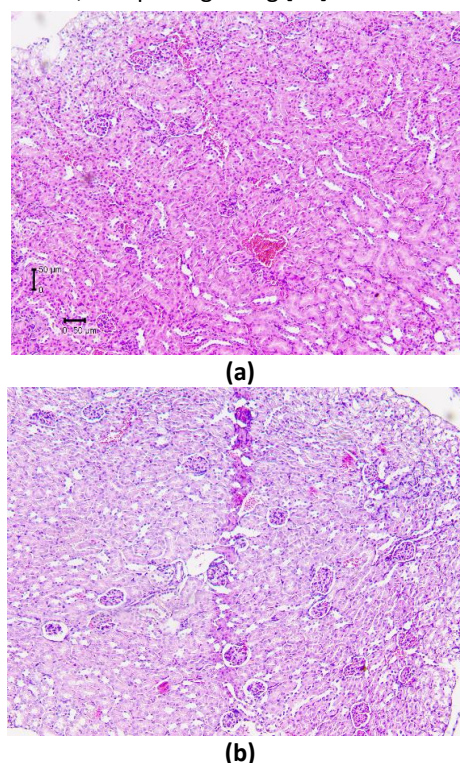


Figure 7. Normal renal histology on control (a) and cancer (b) treatment at 100× magnification. The bar at the bottom left represent 50 μm.

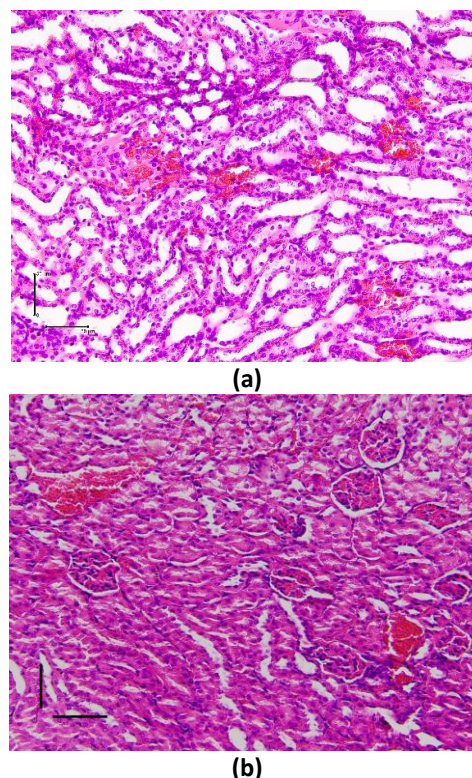


Figure 8. Renal histology on cisplatin treatment at 200× (a,b) magnification. The bar at the bottom left to represent 50 μm.

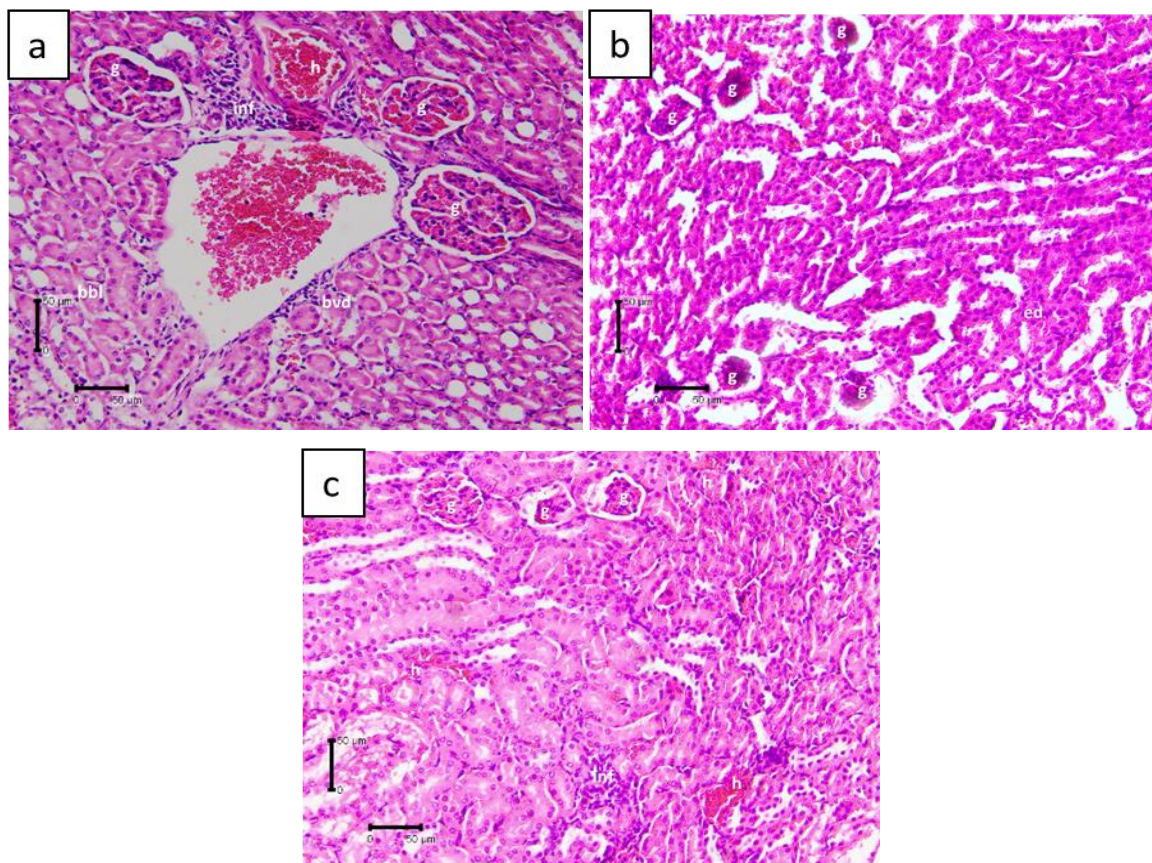


Figure 9. Renal histology on doses 1 (a), 2 (b), and 3 (c) of *Phaleria macrocarpa* extract treatment at 200× magnification. The bar at the bottom left to represent 50 µm.

(Notes: inf= inflammation, h= hemorrhage, g= glomerulus, bvd= blood vessel dilatation, bbl= brush border loss)

In dose 1, even though the glomerulus did not undergo structural changes, tubule inflammation, haemorrhage, and dilation of blood vessels were seen. In doses 2 and 3, there was not much damage to the tubules area but more to the glomerulus. Renal impairment may occur as a direct adverse effect of a metabolite or xenobiotic, specifically in the glomerular area of the tubules (Fig. 9). The cellular mechanism of renal pathogenesis is varied as the wide variety of agents that induce it, including oxidative stress, effect on ion homeostasis, cytoskeletal and mitochondrial injury, lysosomal accumulation and breakdown, and inactivation of signalling kinase [34]. The kidney histology results showed that the plant extract dose does not affect the tissue.

In the glomerular region of the kidney, several glomeruli showed atrophic changes, widening Bowman's space with obvious degeneration of cells. It loses the prominent glomerular structure suggesting apoptotic cell death [35]. Dilation of Bowman's space may occur as a consequence of increased hydrostatic pressure within Bowman's capsule due to

glomerular hyperfiltration or as a consequence of shrinkage of the capillary tufts due to atrophy [34].

Hemorrhage often accompanies acute injury and can occur in the kidney as a primary lesion associated with nephrotoxicants without significant degeneration or necrosis. The presence of luminal hemorrhage implies either damage to the interstitial vascular supply and epithelial basal lamina or damage to the glomeruli as intact erythrocytes do not pass functioning glomerular filtration barriers. The inflammatory reaction is a vital body mechanism to transfer fluid from plasma protein and leukocytes to the tissues in response to injury. This injury can be caused by many factors, including excreted toxic substances or pathogen infection [36]. However, inflammatory cell infiltrates are extremely common in rats and mice and often have no toxicologic significance. The number of inflammatory cell foci increases with age and/or with the presence of chronic nephropathy characteristics [34].

CONCLUSION

Phaleria macrocarpa leaf extract at a dose between 58.9 mg.kg⁻¹ to 117.8 mg.kg⁻¹ BW is optimal for breast cancer treatment because it can reduce tumor volume by more than 90% after three weeks of treatment. Histopathology analysis showed these doses does not cause significant damage to the kidneys tissue, while liver tissue damage can be minimized because it has hepatoprotective activity.

Acknowledgement

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Analysis of Microplastics in Water and Biofilm Matrices in Lahor Reservoirs, East Java, Indonesia

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Abstract

One of the aquatic ecosystems that are very susceptible to contamination is the reservoir ecosystem. Reservoirs have different characteristics from other water bodies because they receive continuous water input from the river that flows through them. The river water contains organic and inorganic materials that can cause pollution caused by various kinds of human activities. The pollutant that is currently getting more attention is microplastics. Hence, various ways are developed to monitor the presence of microplastics in environments. The biotic component that may adsorb and accumulate microplastics is microbes that formed biofilm matrices as a predominant habitat. This study analyzes the presence of microplastics in water and biofilm matrices in Lahor Reservoir. The water parameters (pH, dissolved oxygen, temperature, and flow velocity) were also measured. Samplings were carried out at three stations, namely station A (inlet channel), station B (middle), and station C (outlet channel). This study revealed that water quality parameters were still relatively good according to the environment's quality standards. The average value of microplastic abundance in water at station A was 0.0013 particles.mL⁻¹, station B was 0.00083 particles.mL⁻¹, and station C was 0.00072 particles.mL⁻¹. The average abundance of microplastics in the biofilm at station A, station B, and station C was 7.55 particles.g⁻¹, 7.26 particles.g⁻¹, and 4.59 particles.gram⁻¹, respectively. This study indicates that the abundance of microplastics in the biofilm in the Lahor Reservoir was thousands of times higher than in the water. According to the results of this study, the biofilm can be used as a biological agent in monitoring the presence of microplastics in aquatic ecosystems such as the Lahor Reservoir, East Java, Indonesia.

Keywords: Aquatic Ecosystem, Biofilm, Microbial Ecology, Microplastics, Water Pollutant.

INTRODUCTION

The increase in population and urban development promote the change in people's consumption patterns. Along with the increasingly limited land for activities, the increase in human activities will put more significant pressure on the environment. Human activities in meeting their daily needs, such as agriculture, industry, and household activities, can produce waste that contributes to the decline in water quality [1].

One of the aquatic ecosystems prone to decreasing water quality is the reservoir ecosystem. A reservoir is an example of artificial freshwater created by damming a river. One of the reservoirs in Indonesia is the Lahor Reservoir. This reservoir is part of the Brantas River area development project, carried out in an integrated manner by the Brantas Project Agency. Lahor Reservoir is fed by three rivers: the Lahor River, the Leso River, and the Dewi River. Reservoirs have different characteristics from other water bodies because they receive continuous water

input from the river that flows through them [2]. This river water contains organic and inorganic materials, which have great potential to cause pollution caused by various kinds of human activities [3]. One of the pollutants that are currently getting more attention is microplastics [4].

Microplastics are microscopic plastics found in all of the world's oceans, from beaches and coastlines to subtropical oceans, polar ice caps, and even the deepest parts of the oceans. The size of microplastics is less than 5 mm. However, the lower limit of the particle size included in the microplastic group has not been defined with certainty. The minimum limit often used as a microplastic size is 300 µm [5]. The existence of microplastics can provide various impacts, such as pollution. The impact of microplastic pollution can affect aquatic biota, enter the food chain, and ultimately impact human health. Microplastics can also be carriers of other harmful contaminants, both inorganic and organic [6].

Various attempts have been made to monitor the presence of microplastic contaminants in the ecosystem. One method that can be an alternative is biomonitoring [7]. Biomonitoring is

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a method for monitoring pollutants in an ecosystem using biological agents. The selection of biological agents is one of the primary keys to success in biomonitoring. Biological agents in biomonitoring must be able to accumulate pollutants effectively, easily found in aquatic ecosystems, and easily formed [8,9].

Biomonitoring technology has been developed using biofilms. Biofilms are the dominant form of microbial habitat in aquatic ecosystems [10]. Almost all microbes (> 95%) that live in nature live by forming biofilms [11]. Biofilms are abundant in the aquatic environment and play a variety of essential functions, including the accumulation and purification of pollutants [12,13]. This study used natural biofilm matrices composed of various microbes, such as bacteria, diatom, and algae [14]. The biofilm matrices can be used as biomonitoring agents for several heavy metals such as Pb, Cu, and Cr, Zn in aquatic ecosystems. However, research related to biofilms as biomonitoring microplastics in aquatic ecosystems is still scarce. However, several studies propose that biofilm can accumulate or adsorb microplastics from aquatic ecosystems [15]. Hence, the biofilms showed the potential of biomonitoring agents to monitor microplastics in aquatic ecosystems.

This study aimed to analyze the microplastic content in the water and the biofilm matrix in the Lahor Reservoir. The results of this study were expected as essential knowledge in the use of biofilm as a biomonitoring agent for microplastic pollution in aquatic ecosystems.

MATERIAL AND METHOD

Sampling Area

This research was conducted in the Lahor Reservoir, Sumberpucung Sub-district, Malang Regency, East Java Province, in September 2021. The Lahor Reservoir was built in 1972, has been operating since November 1977, and is part of the Brantas River Basin development project implemented in an integrated manner by the Main Implementing Agency for the Development of the Brantas River Basin.

The Lahor Reservoir is fed by three rivers: the Lahor River, the Leso River, and the Dewi River. Lahor Reservoir has an area of 260 Ha [2]. Water samples in this study were taken from three station points (Fig. 1). Station A is the channel near the inlet (inflow area) of the Lahor Reservoir. Station B is the middle part of the Lahor Reservoir. Station C is the channel near the outlet (outflow area) of the Lahor Reservoir. At each station, the sample was repeated three times. This study used the purposive sampling method to obtain various microplastics.

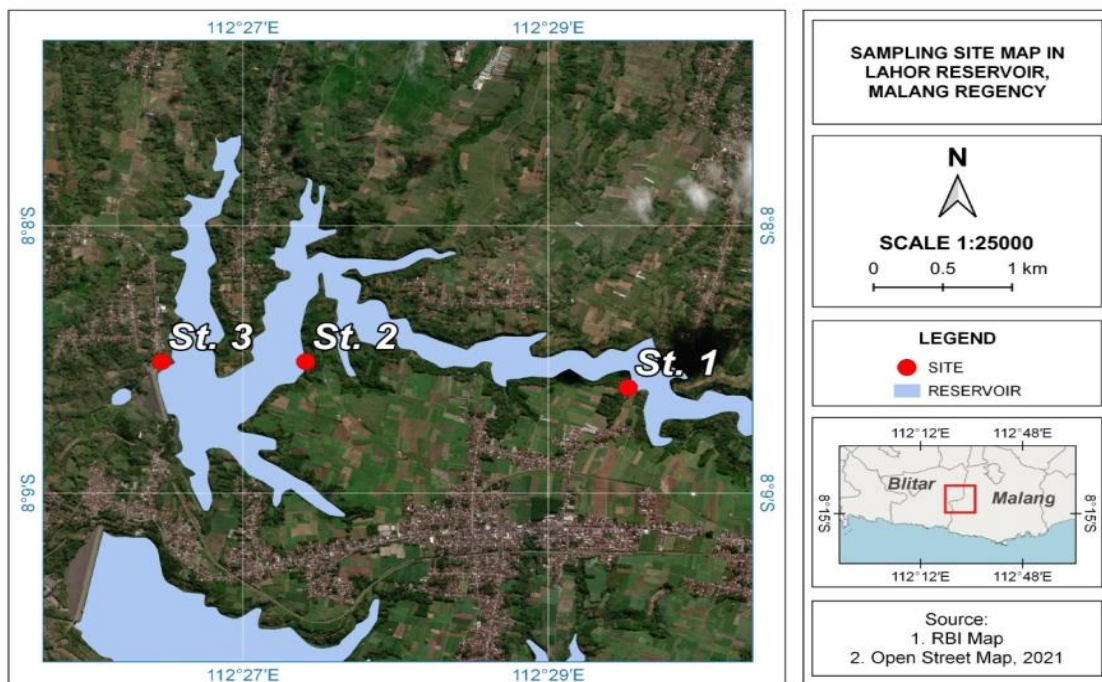


Figure 1. Sampling location

Sampling Procedure

The samples in this study were water and biofilm taken from the Lahor Reservoir. Water samples were taken by filtering 16 L of reservoir water to 250 mL using a plankton net. The plankton net was rinsed with water after all the water samples were filtered so that no microplastic was left on the plankton net. The biofilm used in this study is a biofilm that naturally grows on rocks in the Lahor Reservoir. The biofilm on the rock surface was taken using the brushing method and suspended in 50 mL of sterilized distilled water [16]. Water and biofilm samples were stored in labeled sample bottles and put in a coolbox ($\pm 4^\circ\text{C}$) for analysis in the laboratory.

Water Parameter Measurement

The environmental parameters measured in this study were temperature, pH, dissolved oxygen (DO), and water flow velocity. The temperature was measured using a thermometer. pH was measured using a Lutron PH-201 pH meter. Dissolved Oxygen (DO) was measured using a DO meter Lutron DO-5509. Water velocity was measured using JDC Flowwatch (FL-03) Flowmeter.

Microplastics Analysis

The identification process of microplastic particles taken from water and biofilm in Lahor Reservoir, Sumberpucung sub-district, Malang Regency, East Java Province, was carried out using a modified method based on Masura et al. [17]. The first stage is a wet filtering process to obtain microplastic samples <5 mm using stacked 5 mm and 0.3 mm stainless steel mesh sieves.

The second stage was carried out by oxidizing water samples with the Wet Peroxide Oxidation (WPO) method or wet peroxide oxidation. It aims to remove organic material contaminants in the sample that interfere with the identification and characterization of microplastics. This step was carried out by adding 20 mL of 0.05 M $\text{Fe}_2\text{SO}_4 \cdot 2\text{H}_2\text{O}$ solution to separate the microplastic sample from the metal, then 20 mL of H_2O_2 to dissolve the organic matter.

The mixture of solutions and samples was heated to 75°C on a hot plate to boil for ± 30 minutes. In the case of biofilm samples, after the WPO process was carried out, 6 g of NaCl . 20 mL⁻¹ of the sample was added to increase the density. The separation of organic matter and microplastics in biofilm samples was done using a density separator.

After the process, the third step was to filter the sample by vacuum filtration using Whatman filter paper. After the filtration process, Whatman filter paper was allowed to dry with the help of an oven at a temperature of $\pm 90^\circ\text{C}$.

The fourth stage was to identify microplastics filtered on Whatman paper filter media based on size, shape, and color through microscopic observation. Finally, the abundance of microplastics was calculated and shown as identified particles per volume that was calculated based on the below equation:

$$\text{Microplastics abundance} = \frac{\text{Particle of microplastics observed (particle)}}{\text{Volume of water (mL)}}$$

RESULT AND DISCUSSION

Water Parameters

The environmental parameters measured were temperature, pH, DO, and water flow velocity in this study. These parameters are measured because they can affect the abundance and distribution of microplastics in the aquatic environment. The results of the measurement of these environmental parameters can be seen in Table 1.

The water temperature range of Lahor Reservoir was $28\text{-}30^\circ\text{C}$, pH ranges from 7.6 – 8.5, DO is 7.7 – 8.3 mg.L⁻¹, and current velocity is 0.06 – 1 m.s⁻¹. The water temperature in the Lahor Reservoir shows a value that is classified as optimum for the growth of organisms, including biofilm-forming microbes in the aquatic environment, which is $\pm 30^\circ\text{C}$ [3].

The power of hydrogen is one of the environmental parameters that are very influential on the life of organisms in the waters, especially bacteria. A good environment usually has a pH value ranging from 6.5-8, while the ideal pH value in waters is 7-8.5. Concerning microplastics, the pH value affects the number of microbes, especially microbes that form biofilms and degrade microplastics, so that normal pH conditions in waters can support the life of microplastics degrading or adsorbing microbes [18].

The DO value in the Lahor Reservoir shows an optimum value. The ideal DO value for living organisms in the waters is at least 3 to 7 mg.L⁻¹. Dissolved Oxygen (DO) concentrations in waters also play an essential role in the life of biofilm-forming organisms and microplastic degrading agents. So, DO concentrations in waters will affect the degradation and adsorption processes of microplastics in waters [19].

Table 1. The results of water parameter measurements

No	Stasiun	Temperature (°C)	pH	DO (mg.L ⁻¹)	Water Flow (m.s ⁻¹)
1.	A (Inlet)	28	7.6	7.7	0.06
2.	B (Middle Area)	30	8.5	8.3	0.06
3.	C (Outlet)	28	7.9	8.1	1

Based on the speed of the water flow in the Lahor Reservoir, this reservoir is included in the category of reservoirs with prolonged water flows because the speed is not more than 1 m.s⁻¹. The spread of microplastics in waters depends on several factors, one of which is the speed of water currents. The speed of water currents becomes a medium for transporting microplastics in water. The movement of water makes the number of microplastics uncertain because it continues to change. Water currents will carry microplastic particles from one place to another in the waters. Microplastics will accumulate more in aquatic sediments because the water's currents are generally low at the bottom [20]. The slow speed of water flow makes the possibility of microplastic adsorption by biofilms higher.

Type of Microplastics in Lahor Reservoir

The observed water samples and biofilms found four types of microplastics based on their shape, namely fiber, fragment, film, and bead. The types of microplastics found can be seen in Figure 2. Primary and secondary microplastics dominated the microplastics found in the Lahor Reservoir. Primary sources refer to particles produced in small sizes, such as cosmetics and skin scrubbers. The primary source of microplastics found was in the form of beads. Secondary sources are microplastics produced by the breakdown or fragmentation of larger plastics due to exposure to solar ultraviolet radiation, weathering, or gradual weight loss due to physical damage [19]. The secondary sources of microplastics found were films, fragments, and fibers.

Microplastics' origin and route of entry determine the shape of these microplastics [20]. Fiber microplastics can come from the fragmentation of monofilament fishing nets,

ropes, and synthetic fabrics. Microplastic pollution from anthropogenic sources such as household waste accounts, the largest microplastic is in the form of fragments [21]. The shape of the film has the physical characteristics of being flexible and thin [22]. Film microplastics are thought to come from pieces of degraded single-use plastic bags. Bead-shaped microplastics are produced from the rest of the raw materials for industrial activities, toiletry materials, soaps, and facial cleansers. Microplastics that float with irregular shapes tend to be attracted to water bodies and retained in water bodies [23]. Hence, microplastic particles in films and fragments tend to be found in this study. The previous study had been reported that the general type of microplastics found in the aquatic ecosystem was fragments, fiber, and film [24].

Abundance of Microplastics in Water

The abundance of microplastics in the water surrounding the biofilm in the Lahor Reservoir was analyzed in this study (Figure 3). The average abundance of microplastics in water at station A was 0.0013 particles.mL⁻¹, station B was 0.00083 particles.mL⁻¹, and station C was 0.00072 particles.mL⁻¹. The value of the abundance of microplastics in the waters measured in this study seems to be strongly influenced by the amount of waste input disposed near water sampling time.

The high and low abundance of microplastics in the waters is influenced mainly by the amount of microplastic waste input into the waters. The results of the measurement of the abundance of microplastics in the water presented in Figure 3 show that the highest abundance of microplastics is at the inlet location of the Lahor Reservoir at 0.0013 particles.mL⁻¹ (Station A).

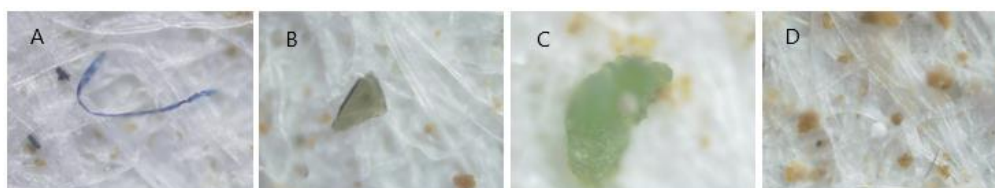


Figure 2. Type of microplastics identified in Lahor Reservoir (A: Fiber, B: Fragment, C: Film, D: Bead)

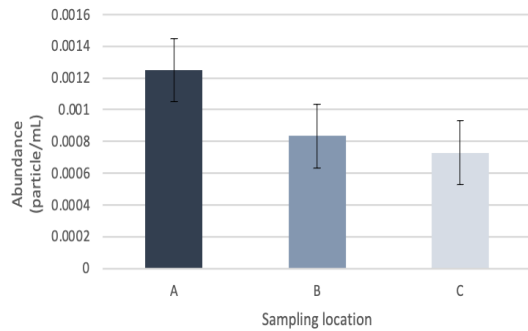


Figure 3. Abundance of microplastics in water of Lahor Reservoir

There was a decrease in the abundance of microplastics in the water along with the increasing distance from the inlet, which is the flow of three rivers, namely the Lahor River, Leso River, and Dewi River. It is suspected that this river flow contains organic and inorganic materials that have great potential to cause pollution caused by various human, domestic activities, anthropogenic activities that produce microplastic pollutants [2].

The second highest abundance of microplastics is in the Lahor Reservoir tourist location, 0.00083 particles.g⁻¹ (Station B). There is a tourist activity in that area that can produce microplastic pollutants. The lowest abundance of microplastics was at the outlet channel of the Lahor Reservoir, which was 0.00072 particles.g⁻¹ (Station C). The liquid waste from Station A will be carried by water flow from Station B to Station C. Along with this flow of water, microplastics can accumulate into various ecosystem components, including biofilms along the river. This accumulation process can result in a decrease in the abundance of microplastics in water as the distance from the input source of microplastic pollutants increases [25].

Abundance of Microplastics in Biofilm

The abundance of microplastics in the biofilm in the Lahor Reservoir was also analyzed in this study (Figure 4). The average value of the abundance of microplastics in the biofilm at station A, station B, and station C were 7.55 particles.g⁻¹, 7.26 particles.g⁻¹, and 4.59 particles.g⁻¹, respectively. These measurements indicate that the abundance of microplastics in the biofilm is thousands times higher than the abundance of microplastics in the water of the Lahor Reservoir. The high abundance of microplastics in the biofilm can be caused by the accumulation of microplastics from water into the biofilm [14]. The accumulation mechanism of

microplastics into biofilms occurred through the interaction of electric charges between biofilms and microplastics [23].

The high abundance of microplastics at stations A and B indicated that the adsorption process of microplastics into the biofilm at this station occurred more than at other stations. This more adsorption could be since the current velocity is slower in this area than in other stations, thus allowing the contact time between microplastics in water and biofilm to be longer. This increased contact time allows more microplastics to accumulate in the biofilm [24].

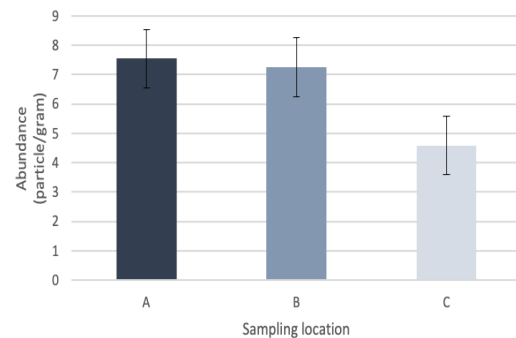


Figure 4. Abundance of microplastics in biofilm matrices formed in Lahor Reservoir

The decrease in the abundance of microplastics at station C maybe because most of the microplastics were tied in the biofilm's interstitial water as the largest part of the biofilm matrix (up to 98%) [26]. Microplastics adsorbed into the biofilm matrix do not completely adhere to the electrically charged sites but can be retained in the inter-polymer space in the biofilm. These results indicate that the microplastics adsorbed in the biofilm are very likely to be released back into the water around the biofilm depending on the abundance of microplastics between the biofilm and water [27]. If the abundance of microplastics between the biofilm and the surrounding water decreases, the microplastics will be desorbed from the biofilm.

Biofilm as Biomonitoring Agent

Based on the comparison of the abundance of microplastics in water and biofilm in the Lahor Reservoir, it is shown that at all stations (assuming 1 mL of water is equivalent to 1 mg) [14], the abundance value of microplastics in biofilms is thousands of times higher than the abundance values of microplastics in water. Therefore, the results of this study indicates that the abundance of microplastics in the biofilm is

more than in water. Under these conditions, microplastics in the biofilm matrix may indicate that the microplastics have been present in reservoir water. Hence, biofilms can be used as biomonitoring agents for microplastic water pollution.

Measurement of the abundance of microplastics in water cannot fully reflect the conditions of pollution or the input of microplastics into the waters. This condition is mainly because, in aquatic ecosystems, the waste disposed of will immediately flow along with the movement of water. Suppose waste disposal is carried out at a difficult time to detect. In that case, it will be difficult for us to detect the presence of discarded waste. In order to evaluate the microplastic content in water, monitoring should represent the occurrence of waste input into the water even though it occurred long before sampling was carried out. Monitoring like this can be done by utilizing ecosystem components in the waters, both biotic and abiotic components [12]. One of the potential alternatives to be developed is the use of biofilms as biomonitoring agents. Plastic-biofilm interactions potentially influence the physical and chemical properties of the polymer, thereby leading to its degradation [28]. Hence, to mitigate the foreseen nuisance of microplastic contamination, biofilm is being looked upon as a potential candidate for remediation and or monitoring of aquatic environments.

CONCLUSION

This study indicates that the abundance of microplastics in the biofilm matrix in the Lahor Reservoir is thousands of times higher than in the reservoir water. It seems that biofilms can accumulate microplastics from the surrounding water. The abundance of microplastics in the biofilm and reservoir water around the biofilm indicates the activity of dumping waste containing microplastics into rivers that flow to the Lahor Reservoir or directly to the Lahor Reservoir. This study indicates that biofilms have the potential to be used as biological agents in monitoring the presence of microplastics in aquatic ecosystems such as the Lahor Reservoir, East Java. In order to develop biofilms in the management of aquatic ecosystems, it is necessary to conduct further research on the level of ability of microplastic accumulation by biofilms in various aquatic ecosystems to monitor microplastic pollutant waste in waters. Further research is needed to determine the adsorption

process of microplastics in biofilms and determine the value of the quality standard for the concentration of microplastics in biofilms as a reference for evaluating the health level of the aquatic ecosystem.

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Effect of Active Detergent Ingredients on Successful Fertilization and Embryo Development of Sea urchins *Tripneustes gratilla* (Linnaeus, 1758)

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Abstract

The success of fertilization and development of sea urchin embryos *Tripneustes gratilla* can be used as a bioindicator of the water quality against the accumulation of pollutants. One of the contaminants that are often used is detergent with an active ingredient in the form of LAS (Linear Alkylbenzene Sulphonate). The purpose of this study was to analyze the effect of LAS (Linear Alkylbenzene Sulphonate) on the success of fertilization and development of sea urchin embryos. This research was conducted in September 2021 at the Zoology Laboratory, Pattimura University, Ambon. *Tripneustes gratilla* were treated with exposure to the active ingredient LAS (Linear Alkylbenzene Sulphonate) with a concentration of 0.0; 0.5; 1, and 2 mg.L⁻¹. The parameter used to assess the success of fertilization is the formation of membrane fertilization. Parameters of embryo development are the division of 2 cells, 4 cells, 8 cells, 16 cells, and 32 cells to form a morula, blastula, and hatching blastula. The results showed that the active ingredient of detergent LAS with a concentration of 0.5 mg.L⁻¹ caused delays in the process of fertilization and embryo development. Meanwhile, the active ingredient LAS with concentrations of 1 and 2 mg.L⁻¹ caused failure in the fertilization process and the embryonic development process of *Tripneustes gratilla* (Linnaeus, 1758).

Keywords: Detergent, embryo, fertilization, *Tripneustes gratilla*.

INTRODUCTION

Detergent is one of the main components of household waste and, in a certain amount, can pollute the aquatic environment because it can cause a lot of foam on the surface of the water [1]. The main component of detergents is surfactants (Surface Active Agents), where surfactants are a type of active ingredient that causes a decrease in the surface tension of the liquid [1]. The most frequently used detergent surfactant is LAS (Linear Alkylbenzene Sulphonate) [2]. One of the aquatic organisms that can be used as a bioindicator of water quality is the sea urchin. It is due to the biological condition of sea urchins that are responsive to changes in environmental conditions [3]. One type of sea urchin that can be used as a bioindicator is *T. gratilla*.

The accumulation of detergent in the sea will cause a lot of foam that can cover the surface of the water, thus disrupting the diffusion of oxygen (O₂) from the air into the water [1]. When the oxygen supply in the water is disturbed, the respiration process in aquatic organisms will also be disrupted [4].

Dissolved oxygen comes from the diffusion process and the photosynthesis process of phytoplankton and is used for respiration by

aquatic organisms and for the decomposition of organic substances by microorganisms [5]. The dissolved oxygen level in waters, according to the Minister of Environment, is <5 mg.L⁻¹ [6]. In addition, the concentration of detergent in water also affects the pH, namely, the higher the concentration of detergent in a water, the higher the pH of the water [7]. Detergents have a pH that ranges from 10-12, while the ideal pH for water ranges from 7-8.5 [6]. The degree of acidity (pH) is a description of the concentration of hydrogen ions that accumulate in a liquid [5]. The existence of variations in pH in water affects the life of organisms that live in these waters considerably. For example, the presence of phytoplankton which supported by the availability of nutrients in these waters [8]. Available environmental conditions such as temperature, salinity, dissolved oxygen, pH, and strong currents affect the survival and reproduction of *T. gratilla* [9], such as successful fertilization and embryo development because the environment is a fertilization medium for sea urchins.

Surfactants are molecules with a polar hydrophilic group and a non-polar hydrophobic group so that the hydrophobic component can damage the egg cell membrane and enter the cell while the hydrophilic will dissolve egg protein and fat in water. Damage to the membrane due to the surfactant will cause fluid to enter the egg cell so that the egg cell does not develop, and after that, cell death will occur [10]. In addition,

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surfactants also suppress the work of respiration, namely inhibition of respiratory activity, which results in the disruption of the oxygen uptake process by the eggs so that the eggs are deprived of oxygen and will die [11]. Damage to eggs is indicated by the opening of the egg cell membrane, the color of the egg changes to pale white, and the gelatin capsule that encloses the egg will turn yellow-brown and then harden [10].

Embryos exposed to pollutants will experience disturbances at their developmental stage. It can result in abnormalities in the form of developmental delays, developmental abnormalities and also decreased differentiation in the embryonic layer [12]. Thus, evaluation of the effect of pollutant toxicity can be carried out by identifying abnormalities in sea urchin embryos [13]. Research on the effect of LAS detergent on water quality has been carried out. However, research using eggs and embryos of *T. gratilla* sea urchins as a bioindicator of contaminants of active detergent ingredients in the coastal area of Ambon City has never been carried out. Therefore, it is necessary to conduct a more specific analysis regarding the effect of the active ingredient of LAS detergent on the success of fertilization and development of sea urchin embryos because this understanding can be used as the basis for developing water quality bioindicators. With the research using *T. gratilla* sea urchins from the waters off the coast of Ambon Island as a model animal in the development of water quality bioindicators, it is hoped that the population of *T. gratilla* sea urchins in nature can be monitored in the future.

METHODS

This research was conducted in September 2021 at the Zoology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Pattimura University, Ambon. The treatment design in this study consisted of several concentrations of LAS (Linear Alkylbenzene Sulphonate) surfactant with three replication on each treatment (Table 1).

Table 1. Treatment Group

Code	Treatment
P1	Seawater + no detergent (Control)
P2	Seawater + LAS 0.5 mg.L ⁻¹
P3	Seawater + LAS 1.0 mg.L ⁻¹
P4	Seawater + LAS 2.0 mg.L ⁻¹

Fertilization and Embryo Culture

Sea urchins were obtained from the coastal waters of Latulalat, Ambon-Maluku. The water

are far from residential areas. It also classified as free of industrial activities. Thus, they are not contaminated by pollutants, which are the main factors underlying the selection of these locations.

Sea urchins taken from nature with a body diameter of 75-80 mm [14] were cleaned and injected with 0.5 M KCl solution as much as 1-3 mL through the peristomial membrane to stimulate spawning. After that, the female sea urchins were placed on a beaker containing seawater, while the male sea urchins were placed on an empty beaker for 30 minutes with the oral position facing down. Eggs and sperm that come out are accommodated in a beaker filled with sea water. The aboral side view of male and female sea urchins shortly after spawning is shown in Figure 1.

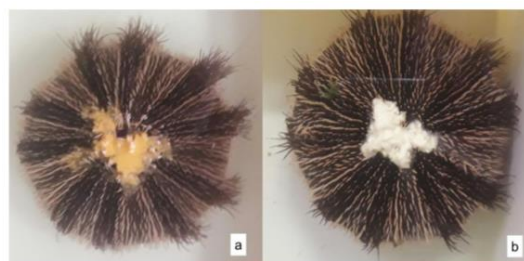


Figure 1. Sea urchin Spawning Aboral View; Oocyte Release (A) and Spermatozoa Release (B) [15]

To analyze the effect of LAS on the success of fertilization, the fertilization process was carried out by mixing 0.1 mL of eggs and 0.1 mL of sperm which had been diluted (0.1 mL of concentrated sperm in 1 mL of seawater). The density of sperm was 1 individual.mL⁻¹ in a petri dish containing 20 mL of seawater and left until the egg is fertilized (embryo phase). Fertilization was carried out in seawater with several different concentrations of LAS (Table 1). Observation of the success of fertilization was carried out 15 minutes after the eggs and sperm were mixed. Eggs were placed in counting columns (Sedgewick Rafter Chambers) to be counted and observed under a microscope with a magnification of 40x.

Furthermore, to analyze the effect of LAS on embryonic development, fertilization was carried out by mixing 0.1 mL of egg and 0.1 mL of sperm which had been diluted (0.1 mL of concentrated sperm in 1 mL of seawater) to reach the embryonic stage in 1000 ml of seawater. After that, the embryos were cultured at a density of 1 individual.mL⁻¹ at different concentrations of LAS (Table 1) in 20 mL of seawater.

The parameters used in this study were the percentage of successful fertilization. The parameters used in this study were the percentage of successful fertilization. It is the number of successfully fertilized eggs indicated by the formation of a fertilization membrane and the percentage of normal embryos. The number of embryos that observed were underwent division starting from 2 cells, 4 cells, 8 cells, 16 cells, 32 cells to form morula, blastula and hatching blastula. Then the embryos were placed in a counting column (Sedgewick Rafter Chambers) and observed under a microscope with a magnification of 40x.

Water Quality Measurement

Water quality was measured for each treatment on each parameter, namely temperature, pH, DO, and salinity. The tools used in this study are a thermometer to measure temperature, a pH meter to measure pH, a DO meter to measure DO, and a refractometer to measure salinity.

Data Analysis

Data in the form of a description of the success of fertilization and embryo development were analyzed descriptively. Data in the form of the percentage of successful fertilization and

embryo development were processed using Microsoft Excel 2013.

RESULT AND DISCUSSION

The Effect of LAS on Fertilization Success

The success of fertilization can be observed based on indications of the formation of a fertilization membrane in the form of a transparent layer that covers the entire outer surface of the egg (Figure 2). The percentage of eggs that were successfully fertilized in the control treatment; 0.5 mg.L⁻¹; 1 mg.L⁻¹ and 2 mg.L⁻¹ were 100%, 7%, 0% and 0%, respectively (Figure 3). It indicates that in the 1 mg.L⁻¹ treatment and 2 mg.L⁻¹ treatment, the eggs were not fertilized, which was characterized by the absence of a fertilization membrane. The environmental conditions available in the control treatment (0.0 mg.L⁻¹) allowed the fertilization process to take place without any disturbance so that the eggs were completely fertilized. The fusion of spermatozoa and egg takes 15 minutes. During the fertilization process, the movement of the cytoplasm increases so that the cell surface becomes irregular. Shortly before the first division begins, the membrane will stop vibration so that the cell surface becomes regular and the hyaline layer thickens [16].

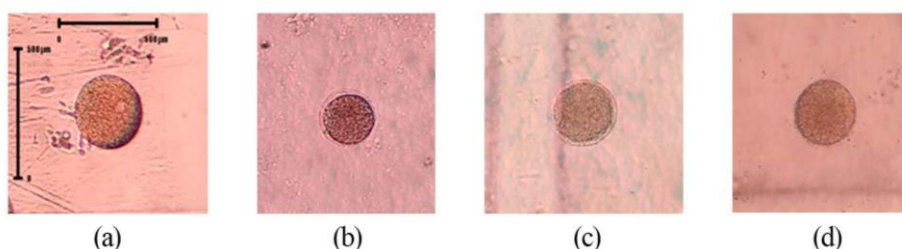


Figure 2. Sea urchin eggs *Tripneustes gratilla* in control treatment, treatment 0.5 mg.L⁻¹ LAS (P2), treatment 1 mg.L⁻¹ LAS (P3) and treatment 2 mg.L⁻¹ LAS (P4). Eggs before fertilization (a); eggs with immature fertilization membranes (b); eggs with complete fertilization membranes (c), and unfertilized eggs (d).

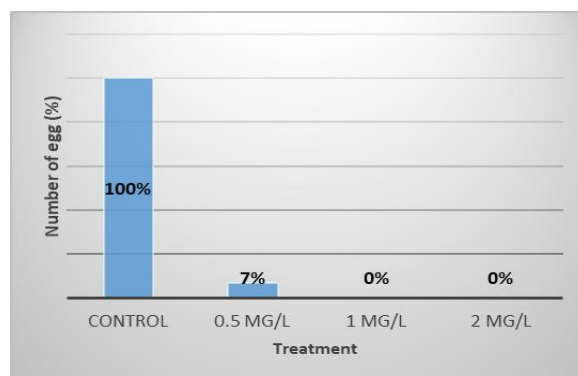


Figure 3. The results of observations of fertilization of *Tripneustes gratilla* sea urchins in the control treatment, 0.5 mg.L⁻¹ LAS treatment, 1 mg.L⁻¹ LAS treatment, and 2 mg.L⁻¹ LAS treatment.

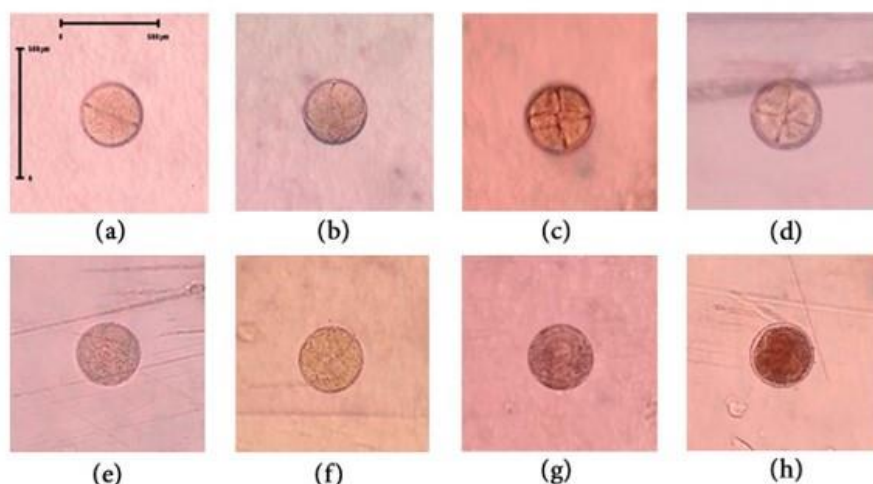


Figure 5. Embryos that failed to develop and embryos that died after being treated with 0.5 mg/L LAS (P2), 1 mg/L LAS (P3) and 2 mg/L LAS (P4): embryos that failed to divide 2 cells (a); embryos that fail to divide 4 cells (b); embryos that fail to divide 8 cells (c); embryos that fail to divide 16 cells (d); 32 cell (e) failed embryos; embryos that fail to morula (f); dead morula (g); dead blastula (h).

Furthermore, at a concentration of 0.5 mg.L⁻¹ the percentage of successful fertilization was 7%, which means that most of the eggs were not fertilized. Unfertilized eggs are eggs that experience a delay in the fertilization process in the form of incomplete membrane formation (Figure 4). LAS at a concentration of mg.L⁻¹ has started to have an effect on egg mortality [11]. LAS quality standard in a waters is 0.5 mg.L⁻¹ [17]. LAS with a concentration of 1 mg.L⁻¹ and 2 mg.L⁻¹ caused damage to the cell membrane due to the interaction between the cell membrane and the LAS hydroxyl group so that the egg cells were penetrated by the LAS. The hydrophobic component of the surfactant damages the egg cell membrane and enters the cell, while the hydrophilic component of the surfactant dissolves egg protein and fat in water [10]. Damage to the membrane will cause fluid to enter the egg so that the egg is not fertilized. Fertilization occurs when the spermatozoa move closer to the jelly layer of the egg cell then the acrosome membrane will fuse with the spermatozoa plasma membrane and exocytosis occurs resulting in the release of enzymes from the acrosome granules. This enzyme allows spermatozoa to penetrate the jelly layer of the egg.

Effect of LAS on Embryo Development

The process of embryonic development begins after the egg is successfully fertilized. Under normal conditions, embryogenesis takes place over 8 hours 45 minutes, starting with the

cell division process until it reaches the morula stage and ends at the blastula stage (Table 2).

Table 2. Embryonic Development Stage

Time Duration	Embryo Stage	Treatment	%
1 h, 12 min	2-cells	control	97
		0.5 mg.L ⁻¹	8
		1.0 mg.L ⁻¹	0
		2.0 mg.L ⁻¹	0
2 h, 3 min	4-cells	control	95
		0.5 mg.L ⁻¹	13
		1.0 mg.L ⁻¹	0
		2.0 mg.L ⁻¹	0
2 h, 28 min	8-cells	control	95
		0.5 mg.L ⁻¹	10
		1.0 mg.L ⁻¹	0
		2.0 mg.L ⁻¹	0
2 h, 51 min	16-cells	control	95
		0.5 mg.L ⁻¹	0
		1.0 mg.L ⁻¹	0
		2.0 mg.L ⁻¹	0
3 h, 12 min	32-cells	control	95
		0.5 mg.L ⁻¹	0
		1.0 mg.L ⁻¹	0
		2.0 mg.L ⁻¹	0
3 h, 32 min	morula	control	95
		0.5 mg.L ⁻¹	0
		1.0 mg.L ⁻¹	0
		2.0 mg.L ⁻¹	0
8 h, 45 min	blastula	control	94
		0.5 mg.L ⁻¹	0
		1.0 mg.L ⁻¹	0
		2.0 mg.L ⁻¹	0

Note: Time duration after fertilization; h=hour, min=minutes. At the blastula stage, the embryo hatches from the fertilization membrane that encloses it during embryogenesis.

In the control treatment, the embryos developed successfully under normal conditions. However, in the 0.5 mg.L⁻¹ treatment, the embryos experienced developmental delays at the 2-cells, 4-cells, 8-cells stages, and died at the 16-cells, 32-cells, morula, and blastula stages. Accumulated LAS concentrations exceeding 0.5 mg.L⁻¹ are toxic to various aquatic organisms [17]. It is in line with the results proposed by a previous study where at a concentration of 0.5 mg.L⁻¹, there were abnormalities in eggs caused by the entry of surfactants through the egg membrane resulting in chromosomal abnormalities and gene damage. Certain concentration of surfactants could denature proteins. It will damage several enzymes and hormone systems involved in embryonic development [11]. LAS threshold standard in a water is 0.5 mg.L⁻¹ [17].

Meanwhile, in the 1 mg.L⁻¹ treatment and 2 mg.L⁻¹ treatment, the embryo failed to develop and died at each stage of its development. An increase in the concentration of LAS 1 mg.L⁻¹ and 2 mg.L⁻¹ causes the activation of hatching enzymes such as chorionase enzymes which are inhibited so that embryos fail to hatch, besides that the embryos also fail to divide, are disabled, and die [3]. The egg mortality rate increased along with the increase in LAS concentration as reported in previous studies, namely at a concentration of 1.5 mg.L⁻¹ and 3 mg.L⁻¹, eggs died which was marked by a change in egg color which was initially bright or transparent then turned white or brown cloudy [11].

Culture Media Water Quality

The quality of the culture media was measured during the culture process, which included several parameters, namely temperature, pH, DO, and salinity. The results of water quality measurements in culture media showed that the temperature of the culture media increased with the increase in the concentration of the active ingredient of LAS detergent, which resulted in a greater percentage of embryo mortality. Temperature greatly affects the development of the short planktonic period of sea urchins [18]. When the ambient temperature becomes higher, then the hatchability will also increase. Otherwise, if the ambient temperature becomes lower, then the hatchability will also decrease [19]. An increase in temperature will increase the rate of respiration so that the need for oxygen will also increase. Respiration results in an increase in carbon

dioxide in seawater due to reacting with acidic carbonic acid, so that the pH value will also decrease. A decrease in the pH value affects sperm movement, which becomes slower [20].

Table 2. Culture media water quality

LAS (mg.L ⁻¹)	Temperature (°C)	pH	DO (mg.L ⁻¹)	Salinity (ppt)
0*	26.6	7.62	3.0	35.0
0.5	26.7	7.59	3.1	34.4
1	26.8	7.57	3.2	34.2
2	26.9	7.56	3.2	34.0

Note: *control (seawater, no LAS).

Furthermore, dissolved oxygen (DO) levels in the culture media increased in line with the increase in the concentration of the active ingredient in LAS detergent. The increase in DO levels was due to an increase in the percentage of embryonic death, so that the embryo's need for oxygen was also lower. Then the salinity in the culture media decreased when the concentration of the active ingredient in LAS detergent increased. Echinoderms are generally not resistant to low salinity except for species that live in tidal areas, such as sea urchins [21]. The salinity of marine waters ranges from 30-40 ppt [22].

CONCLUSION

The active ingredient of detergent LAS (Linear Alkylbenzene Sulphonate) with a concentration of 0.5 mg.L⁻¹ caused delays in the process of fertilization and embryo development. Meanwhile, the active ingredient LAS (Linear Alkylbenzene Sulphonate) with concentrations of 1 mg.L⁻¹ and 2 mg.L⁻¹ caused failure in the fertilization process and the embryonic development process of *Tripneustes gratilla*.

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Expression of IL-17 on Breast Cancer Mice Treated by Combination of *Phyllanthus Urinaria* and *Catharanthus roseus* Extract

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Abstract

This study aimed to evaluate the effect of combination *Phyllanthus urinaria* and *Catharanthus roseus* in breast cancer mice based on the expression of IL-17. 7,12-Dimethylbenz(a)anthracene (DMBA) was injected intraperitoneally into normal mice at dose 1.5 mg.kg⁻¹ weight to obtain breast cancer mice. Total of 24 experimental mice divided into normal mice (N), breast cancer mice (K), breast cancer mice with cisplatin (C) treatment (5 mg.kg⁻¹ weight), breast cancer mice with combination extract Dose 1 (*P. urinaria* 500 mg.kg⁻¹ weight + *C. roseus* 15mg.kg⁻¹ weight), breast cancer mice with combination extract Dose 2 (*P. urinaria* 1000 mg.kg⁻¹ weight + *C. roseus* 75mg.kg⁻¹ weight), and breast cancer with combination extract Dose 3 (*P. urinaria* 2000 mg.kg⁻¹ weight + *C. roseus* 375 mg.kg⁻¹ weight). Cheral was given orally for 14 days. The level of IL-17 was evaluated by flow cytometry analysis. The combination can suppress the expression of IL-17 which down regulation of IL-17 indicate a good prognosis for the breast cancer mice, for 6.17% in breast cancer condition to 0.93% with Dose 3 treatment. The combination can be used as immunomodulatory agent in humoral immunity through the regulation of IL-17.

Keywords: Breast cancer, *Catharanthus roseus*, IL-17, *Phyllanthus urinaria*

INTRODUCTION

The research of cancer has developed in carcinogenesis and cancer genetics based on molecular and cellular. It is known that cancer is one of the leading causes of death in the world. One of the most deadly types of cancer among women is breast cancer, intestinal cancer and lung cancer [1]. Various studies show that 1 in 8 women have breast cancer where 90% of the causes of breast cancer are due to lifestyle and environmental factors, while 10% of breast cancer cases are caused by genetic factors [2]. Researchers have predicted that there will be an increase in breast cancer cases, with death cases up to 58% in developing countries [3]. Chemotherapy with surgery is the most common treatment for breast cancer cases. However, chemotherapy treatment has many side effects in people with breast cancer. So more research needs to be done to find new strategies in the treatment of cancer [4].

In the tumor microenvironment, it is known that accumulated tumor cells will inhibit the immune response by various mechanisms [5]. One of the mechanisms of tumor cells inhibiting the immune response is to develop inflammatory conditions in the tumor micro-environment, where tumor cells will cause inflammation by

infiltrating leukocytes and secreting inflammatory cytokines at tumor locations [6-8]. Several studies have shown the role of inflammatory cells and cytokines accumulated in the tumor microenvironment can suppress the immune response and contribute to the growth and development of tumor cells [4].

One of the proinflammatory cytokines that play a role in the development and growth of cancer and inhibition of the immune system is IL-17 consisting of IL-17A and IL-17F. IL-17 are proinflammatory cytokines derived from CD4⁺ T cells that function as both homodimers and heterodimers and play a role in signaling through heterodimeric receptor complexes consisting of IL-17 receptor A (IL-17RA) and IL-17RC [9]. IL-17 is also known to be one of the main pathogen factors involved in the early and late stages of cancer development. Removal of IL-17 was able to suppress tumorigenesis in various organs in mice including the large intestine [10], liver [11], pancreas [12], lungs [13], and skin [14]. Inhibition of IL-17 cytokines has been shown to suppress metastasis and increase sensitivity to chemotherapy and radiation therapy in preclinical cancer models [15]. High serum levels of IL-17 indicate a poor prognosis for various tumors in people with cancer [16]. Some polymorphisms against IL-17 have been known to have a link to cancer [17-19].

The development of cancer drugs has been done to get the best treatment for cancer. Type of drug based on its toxicity to DNA inhibitors (deoxyribonucleic acid), such as cytosine

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arabinoside and methotrexate drugs. Then also based on alkylation agents such as the drugs cisplatin and 5-fluorouracil. Chemotherapy is a conventional treatment to overcome cancer malignancy [20]. However, chemotherapy treatment is known to have many side effects on breast cancer patients. So herbal treatment is needed to minimize the negative impact of treatment. Based on the literature, *Phyllanthus urinaria* can induce apoptotic in melanoma cancer cells without any cytotoxic effect in normal cell lines [21].

Green Meniran (*Phyllanthus urinaria*) is one of the plants that has been known to have several biological effects such as antibacterial, antiviral, immunoregulation, and anti-inflammatory [22]. *Phyllanthus urinaria* also exhibits anticancer properties in melanoma, osteosarcoma, lung cancer, breast cancer, and prostate cells [23]. This plant has been shown to inhibit tumor cell proliferation through induction of apoptosis by modulating various cell signaling pathways [21]. In addition to *P. urinaria*, Tapak Dara (*Catharanthus roseus*) is also known to have a role as an anticancer. *Catharanthus roseus* is a plant containing more than 120 terpenoid alkaloids (TIA). One of the alkaloids in *C. roseus* is vincristine and vinblastine, which act as antineoplastics and are often used as anticancer agents [24]. The combination of both is expected to suppress the growth of breast cancer at the level of IL-17 expressed by CD4⁺. The aim of the study was to evaluate the level of IL-17 expressed by CD4⁺ in breast cancer mice.

MATERIAL AND METHODS

Design of Experimental

Total mice of experimental are 24 female mice (*Mus musculus*) strain BALB/c with age of 6-7 weeks from Gadjah Mada University, Yogyakarta, Indonesia. The mice divide into six groups (Table 1). Each group consisted of four replication. This experiment has been approved by ethical clearance by The Ethical Committee of Brawijaya University (Reg. No. 125-KEP-UB-2021).

Table 1. Group of treatment

Group Description	Combination Extract (mg.kg ⁻¹ weight)	
	<i>P. urinaria</i>	<i>C. roseus</i>
N Normal mice	-	-
K Cancer mice	-	-
C Cancer mice + cisplatin	-	-
D1 Cancer mice	500	15
D2 Cancer mice	1000	75
D3 Cancer mice	2000	375

Note: D = dose

Induction of DMBA

Mice induced by 7.12- Dimethylbenz(α) anthracene 1G cat. D464500 TRC (DMBA) with 1.5 mg.kg⁻¹ weight, which the dose was convert as FDA table [15]. DMBA was dissolved in corn oil and injected into mice subcutaneous six times in six weeks. Confirmation of breast cancer condition used palpasi method and histology.

Oral Administration of Extract Combination and Cisplatin

Ten grams of Green Meniran (*P. urinaria*) and ten grams of Tapak Dara (*C. roseus*) leaves powder (Materia Medica Batu, Malang) dissolved in 100 mL of boiled water. Then it was filtered using Whatman no. 1 paper and subjected to freeze-drying. The extract combination was dissolved by aquadest and administrated in breast cancer mice (D1, D2, and D3) orally for 14 days.

Cisplatin (Cis-Diammineplatinum (II) Dichloride) H₆Cl₂N₂Pt CAS 15663-27-1 as a standard drug in this study was obtained from the Tokyo Chemical industry. CO. LTD. Cisplatin dissolved with PBS and injected intraperitoneal on mice (5 mg.kg⁻¹ weight). Cisplatin was administrated in the breast cancer group orally for 14 days. The conversion of human dose to mice equivalent dose in this research is determined based on FDA (Food and Drug Administration) administration [25].

Spleen Isolation

Spleen isolation was done after 14 days of extract combination treatment. The mice were sacrificed by the cervical dislocation technique. Then, the spleen was isolated from the mice. The spleen was homogenized in phosphate buffer saline (PBS), and the homogenate was moved into a microtube and centrifuged at 2500 rpm at 10°C for 5 minutes. Then, the pellet suspends with 1 mL PBS as the sample for antibody staining.

Antibody staining and Flow Cytometry Analysis

Antibody staining consists of two kinds membrane staining and intracellular staining. Antibodies applied 50 μL based on the company's protocol. The sample, which contain lymphocyte cells, was stained with extracellular antibodies before intracellular antibody staining. Extracellular antibody staining was conducted by adding 50 μL of FITC anti-mouse CD4⁺ (Biologend®, San Diego) into the cells and incubated for 20 minutes in an icebox (4°C).

The cells were added with 50µL of cytofix (BD-Biosciences Pharmigen) and incubated for 20 minutes in an icebox, then added by wash perm solution (WPS) (Biolegend®, USA). The sample was centrifuged at 2500 rpm at 10°C for 10 minutes. The pellet was stained with 50 µL of anti IL-17 (Biolegend®, San Diego). The sample was moved into the flowcytometry tube by adding 400 µL of PBS. The data of flowcytometry was analyzed by software BD Cellquest Pro™ (BD Biosciences, San Jose, CA, USA) [26].

Data Analysis

Data were analyzed by IBM SPSS Statistic 26 program using one-way ANOVA and continued to Post Hoc test with a significant $p < 0.05$.

RESULT AND DISCUSSION

The results showed a significant increase in the relative amount of IL-17 expressed by CD4+ ($p < 0.05$) compared to healthy controls. The increase of IL-17 expressed by CD4 T cells can be due to the presence of a mechanism of the DMBA compound that can cause tumorigenesis in the tissues, thus causing an increase in the production of the pro-inflammatory cytokine IL-17. DMBA compounds are carcinogenic chemical compounds that are often used to induce mammary carcinogenesis in mice [27]. Some extrahepatic tissues, such as mammary glands, can also occur deposition and activation of hydrophobic compounds such as DMBA. DMBA induced in mice will bind to cytochrome P-450 and form a covalent bond with DNA that will cause DNA adduct, which will cause oxidative damage to the cell resulting in carcinogenicity and mutagenicity [28].

The process of DMBA carcinogenesis will involve various disorders, such as disruption of redox tissue balance. It is also able to cause oxidative stress that is responsible for biochemical and pathophysiological disorders in mice [29]. Oxidative stress with the formation of ROS is known to have a negative impact on a person's health and is associated with various diseases, including cancer. ROS will alter the expression of tumor suppressor genes involved in apoptosis. It can increase the expression of cytokines involved in the angiogenesis process. It creates changes in intercellular relationships and affect the metalloproteinase activity of proteinases involved in metastasis [30]. ROS can also interfere with the integrity of the cell membrane by reacting with PUFA, which will lead to the formation of malondialdehyde (MDA) [28].

An increase in the relative amount of IL-17 expressed by CD4⁺ in the lymph organs indicates that the experimental animal has developed cancer characterized by the production of pro-inflammatory cytokines. These pro-inflammatory cytokines promote the inflammatory part of the affected tissue. IL-17A is also called IL-17 [31], which is a proinflammatory cytokine that has been studied extensively. IL-17 is a cytokine that has various roles by combining IL-17RA-IL-17RC dimers that can be found in various cells. It is commonly known that the cytokine IL-17 is a cytokine produced by Th17 cells that belongs to the CD4⁺ T lymphocyte subgroup. Various chronic inflammatory diseases and autoimmune diseases are known due to the increase in the number of IL-17. Several studies have shown that the number of IL-17A-producing cells increases in cases of breast cancer [30,31].

IL-17A in cases of breast cancer has a direct and indirect influence. The direct effect of IL-17 in tumor cells is able to promote angiogenesis, change the profile of gene expression, and it is able to make cells more aggressive and conducive to tumor growth in vivo [32]. Meanwhile, the indirect effects of IL-17 support the growth of metastatic primary breast cancer [15]. IL-17 can promote cancer growth, control neutrophil recruitment, and recruit neutrophils in secondary areas affected by cancer [15,33].

Based on the results of the study, it is known that there is a decrease in the relative amount of various treatments. A decrease in the relative number of CD4⁺IL-17⁺ T cells in cisplatin and chel treatment doses 1, 2, and 3 indicated a significant decrease in the number of relatives ($p < 0.05$) with amounts of 2%, 1.88%, 2.76%, and 0.93% respectively (Fig. 1). At dose 1, it is shown the chel can suppress the growth of IL-17 cytokines significantly with the lowest dose. However, in dose 2, it is known that there is an increase in the amount of IL-17 compared to Dose 1 treatment. Thus, the addition of doses at dose 2 was not able to suppress the development of IL-17, so there is an increase. However, it is known that at Dose 3, the relative amount of cytokine IL-17 decreased significantly compared to all treatments and was close to the relative amount in normal mice. A decrease in the relative amount of IL-17 at Dose 3 is suspected that the dose given has been able to suppress the growth of IL-17 cytokines resulting in a decrease in the number of IL-17 expressions (Fig. 1).

This research showed that the expression of IL-17 was decreased in cisplatin treatment.

Decreased expression of IL-17 in cisplatin treatment in this study is known that cisplatin is a rectangular planar platinum compound which is one of the potential chemotherapeutic agents in anticancer therapy. Clinically, the anticancer activity of cisplatin acts on various types of cancer, including lung cancer, ovarian cancer, carcinoma, breast cancer, and brain cancer [34]. There are several pathways of cisplatin molecular mechanisms in achieving therapeutic effects, namely through oxidative stress induction, Ca signal modulation, induction of cell apoptosis, protein kinase C, mitogen-activated protein kinase (MAPK), Jun amino-terminal kinase (JNK),

p38 mitogen-activated protein kinase. (MAPK), AKT (serine/threonine kinase), DNA signaling damage, and p53 DNA damage [31]. The use of cisplatin as a neoplastic drug has been approved by the United States Food and Drug Administration (FDA) since 1978. The use of cisplatin dose is given based on the stage of the cancer. A previous study showed that the use of the drug cisplatin can reduce the growth of breast cancer, but it does not rule out the possibility of resistance [20]. Based on this, it is known that our research is in line with some of the studies that have been reported [16].

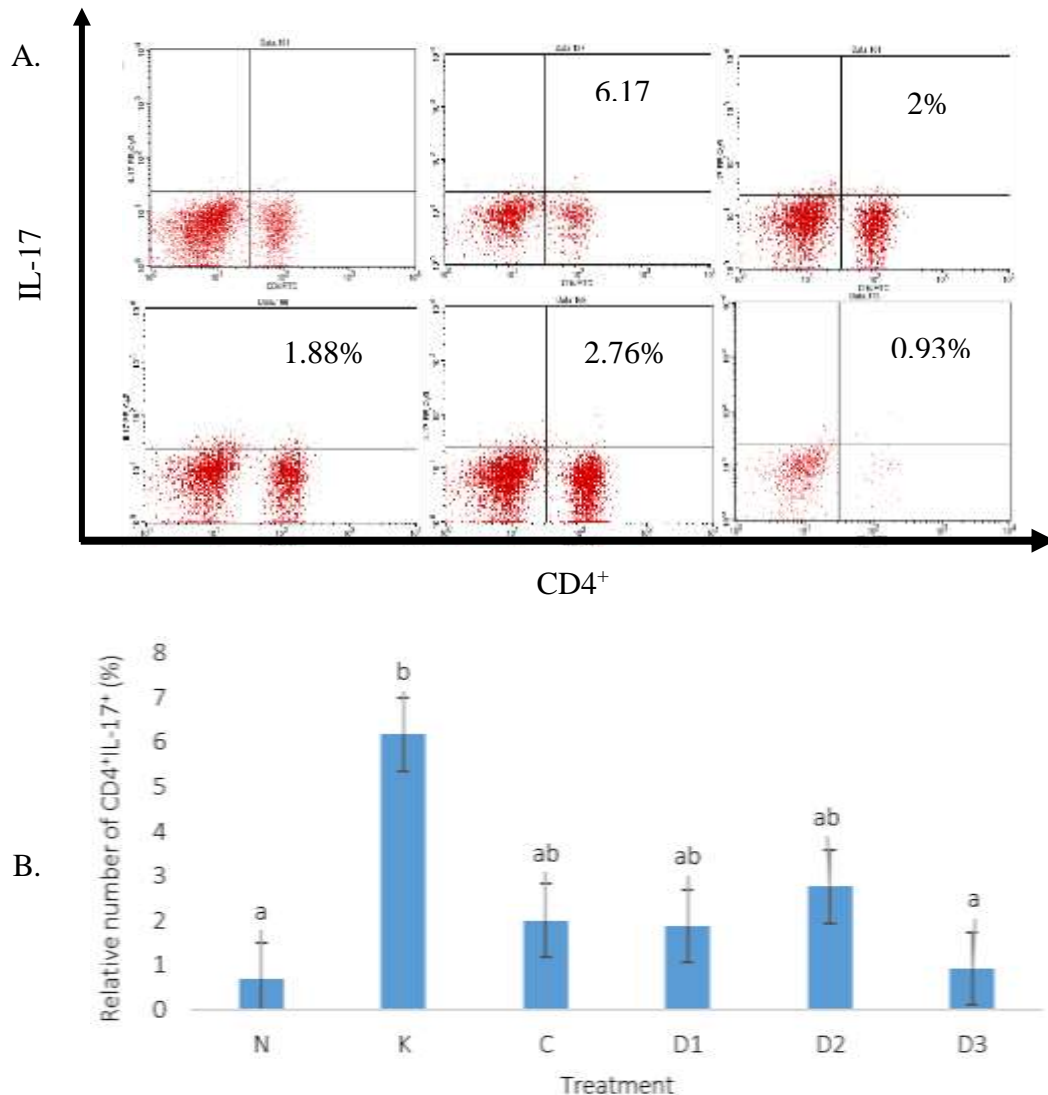


Figure 1. Relative number of CD4+IL-17+ (%) in mice with a wide variety of treatments. N = Healthy control (Normal); K = DMBA injection (cancer control); C = DMBA and Cisplatin injected mice; D1 (dose 1) = DMBA injection and of 500 mg.kg⁻¹ weight *P. urinaria* and 15 mg.kg⁻¹ weight *C. roseus*; D2 = injection of DMBA and 1000 mg.kg⁻¹ weight *P. urinaria* and 75 mg.kg⁻¹ weight *C. roseus*; D3 = injection of DMBA and 2000 mg.kg⁻¹ weight *P. urinaria* and 375 mg.kg⁻¹ weight *C. roseus*; line on bar = standard deviation. A) Results of T cell flow cytometry analysis (%); B) CD4+IL-17+ T-Cells (%) graph results. The data are mean value ± SD of four mice in each group with significant value <0.05 (n=24).

A combination of *P. urinaria* and *C. roseus* can suppress IL-17. IL-17 cytokines are known to be proinflammatory cytokines that support cancer growth. The reduction of the relative amount of IL-17 expressed by CD4⁺ at the 1,2,3 dose treatment can be suspected due to the influence of phytochemicals contained in the content of the combination extract that can reduce the activity of increasing cancer cells. In addition, *P. urinaria* is also able to inhibit activator plasminogen type urokinase and MMP-2 enzyme activity that can inhibit phosphorylation of ERK1/2 [35]. Several studies have shown the role of *P. urinaria* as an anticancer, antioxidant, antiviral, antidiabetic, and anti-inflammatory activity [36].

Several studies *In Vivo* have proven that *P. urinaria* is able to inhibit the development of colorectal cancer in Sparague-Dawley male mice through the perforin granzyme pathway, and it is able to lower proinflammatory cytokines that cause cancer cell growth and macroscopic tumor growth [37]. *Phyllanthus urinaria* research has also been conducted on liver carcinoma which shows the influence of *P. urinaria* which is able to increase apoptosis and suppress the growth of cancer cells. Phase II clinical trials have also been conducted on people with rectum cancer, where the results of clinical trial II showed the extract. *Phyllanthus urnaria* can significantly increase cancer cell apoptosis and expression of lymphocytes, perforins, granzymes, and caspase 3 that infiltrate significantly [38].

In addition to *P. urinaria*, the content of compounds in *C. roseus* is also thought to play a role in the suppression of cancer cell growth characterized by the expression of IL-17. *Catharanthus roseus* contains vincristine and vinblastine compounds that are able to inhibit cancer cells and can inhibit the synthesis of purines, DNA, and RNA contained in cancer cells to inhibit the process of cancer cell proliferation [39]. This plant has been used in traditional medicine. It is known that a number of alkaloids isolated from this plant have been clinically tested.

Alkaloid compounds in *C. roseus* are ajmalicine-an antihypertensive alkaloids, vincristine and vinblastine as antineoplastic. This plant is used in the treatment of cancer because it is able to interfere with cell signals such as growth factor signals, inflammation, cell cycle proteins, prostaglandin production, angiogenesis, invasion, anti-apoptosis, and cell proliferation [40]. The pharmacological study has shown that

C. roseus contains more than 70 types of alkaloids and chemotherapeutic agents that are effectively able to treat various diseases such as lung cancer, uterine cancer, breast cancer, Hodgkin and non-Hodgkin lymphoma, and melanoma [40]. Based on the results of the study, there is a decrease in the number of IL-17 expressions allegedly due to the influence of the two plants that synergize in the treatment of breast cancer in experimental animals.

CONCLUSION

The combination of *P. urinaria* and *C. roseus* could be used as the immunomodulator in breast cancer mice based on the expression of cytokine proinflammatory of IL-17 expressed by CD4⁺. The oral administration of extract combination for 14 days in breast cancer mice significantly decreased the IL-17 expression from a 6.17% to 1.88% (D1), 2.76% (D2), and 0.93% (D3). We can conclude that Dose 3 is the optimal dose to decrease the expression of IL-1.

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Data Collection (Calibri 10 Bold, Left)

Explain the data collection methods, i.e. surveys, observations or archive, accompanied by details of the use of such methods. This section also describes the population, sampling and sample selection methods. (Calibri 10 Justify)

The use of English language should followed proper grammar and terms. Name of organism should be followed by its full scientific name in the first mention, in *italic* [3]. Author of the scientific name and the word of “var.” typed regular. Example: *Stellaria saxatillis* Buch. Ham. First abbreviation typed in colon after the abbreviated phrase.

Author must use International Standard Unit (SI). Negative exponent used to show the denominator unit. Example: g l⁻¹, instead of g/l. The unit spaced after the numbers, except percentage [4]. Example: 25 g l⁻¹, instead of 25gl⁻¹; 35% instead of 35 %. Decimal typed in dot (not coma). All tables and figures should be mentioned in the text.

RESULT AND DISCUSSION (Calibri 10 Bold, Left, Capslock)

This section contains the results of the analysis and interpretation or discussion of the results of the analysis. Describe a structured, detailed, complete and concise explanation, so that the reader can follow the flow of analysis and thinking of researchers [5]. Part of the results study should be integrated with the results of the

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Address : affiliation address include post code

analysis and the results and discussion are not separated.

Table

Table should be submitted within the manuscript and in separated file of *Microsoft Excel* (xls.). Table should not exceed 8 cm (one column) and 17 cm (two columns). Table should be embedded in different page after references.

Table should be numbered in sequence. Table title should be brief and clear above the table, with uppercase in initial sentence. Vertical line should not be used. Footnote use number with colon and superscripted. Symbol of (*) or (**) was used to show difference in confidence interval of 95 and 99%.

Table 1. Example of the Table (Calibri 8.5 Left)

No	Point (Calibri 8.5 Justify)	Description
1		
2		
3		
4		
5		

Sources: Journal of PPSUB (Calibri 8.5 Left)

Figures

Figures should be in high resolution and well contrast in JPEG or PDF with the following conditions:

- Monochrome image (line art), figures of black and white diagram (solid/no shades of gray), resolution 1000-1200 dpi (dot per inch).
- Combination Halftone, combine figure and text (image containing text) and coloured graphic or in grayscale format. Resolution 600-900 dpi.
- Halftone, coloured figure or grayscale format without text. Resolution 300 dpi.

- Black and white figure should be in the grayscale mode, while coloured figures should be in RGB mode.
- Figure should not exceed the width of 8 cm (one column), 12.5 cm (1.5 columns) or 17 cm (two columns).
- Figures title typed clearly below the figure.
- Figure with pointing arrow should be grouped (grouping).
- Figures were recommended in black and white.
- Legend or figure description should be clear and complete. If compressed, the figure should be readable.
- Statistic graphic should be supplemented with data sources.
- If the figures come from the third party, it should have the copyright transfer from the sources.

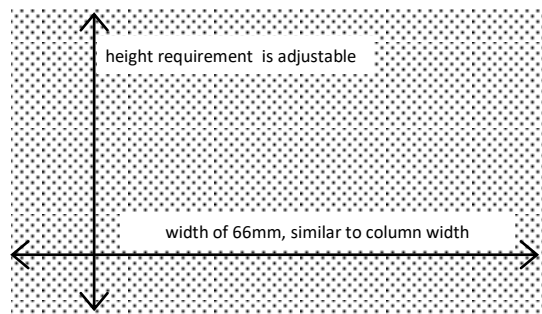


Figure 1. Illustration of Dimensional Figure of one column width. Figure dimension adjusted to the width of one column. Name the figure (diagram) written below the image. (Calibri 8.5 Justify)

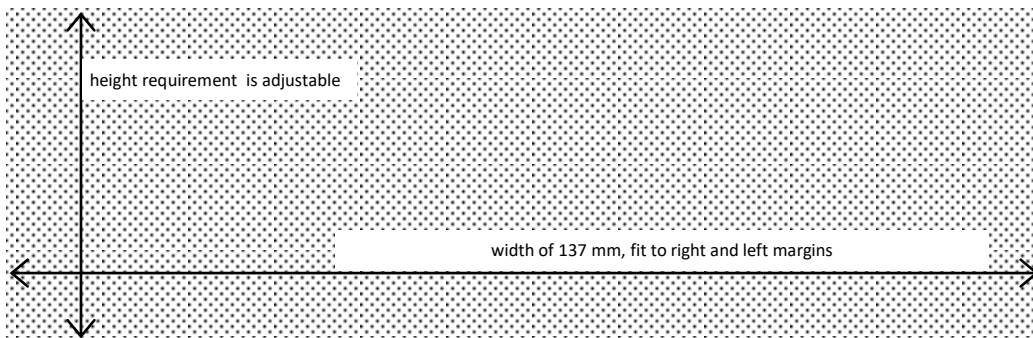


Figure 2. Illustration of Dimensional Figure of two column width. Figure dimension adjusted to the width of two columns (137 mm). Figure were align top or bottom of the page. (Calibri 8.5 Justify)

References

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

- [4].Syafi'i, M., Hakim, L., dan Yanuwiyadi, B. 2010. Potential Analysis of Indigenous Knowledge (IK) in Ngadas Village as Tourism Attraction. pp. 217-234. In: Widodo, Y. Noviantari (eds.) Proceed-ing *Basic Science National Seminar 7* Vol.4. Universitas Brawijaya, Malang. (Article within conference proceeding)
- [5].Dean, R.G. 1990. Freak waves: A possible explanation. p. 1-65. In Torum, A., O.T. Gudmestad (eds). Water wave kinetics. CRC Press. New York. (Chapter in a Book)
- [6].Astuti, A.M. 2008. The Effect of Water Fraction of *Stellaria* sp. on the Content of TNF- α in Mice (*Mus musculus* BALB-C). Thesis. Department of Biology. University of Brawijaya. Malang. (Thesis)

CONCLUSION (Calibri 10 Bold, Left, Capslock)

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

ACKNOWLEDGEMENT (Calibri 10 Bold, Left, Capslock)

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

REFERENCES (Calibri 10 Bold, Left, Capslock)

- [1].(Calibri 10 Justify, citation labelling by references numbering)
- [2].Vander, A., J. Sherman., D. Luciano. 2001. Human Physiology: The Mecanisms of Body Function. McGraw-Hill Higher Education. New York. (Book)
- [3].Shi, Z., M. Rifa'i, Y. Lee, K. Isobe, H. Suzuki. 2007. Importance of CD80/CD86-CD28 interaction in the recognition of target cells by CD8⁺CD122⁺ regulatory T cells. *Journal Immunology*. 124. 1:121-128. (Article in Journal)

Cover Image
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