

JELS

ISSN 2087-2852

A large, stylized molecular structure visualization occupies the top half of the cover. It features a complex network of green and red spheres connected by lines, set against a blue and purple background with a grid of small dots. The structure appears to be a complex organic molecule, possibly a protein or a large polymer, with a central core and branching structures.

The Journal of **EXPERIMENTAL** **LIFE SCIENCE**

J.Exp. Life Sci.	Vol.12	No.1	Pages. 1-38	February 2022
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Published by :
Graduate Program, Universitas Brawijaya

jels.ub.ac.id

The Journal of **Experimental** *Life Science*

Discovering Living System Concept through Nano, Molecular and Cellular Biology

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DOI: <https://doi.org/10.21776/ub.jels.2022.012.01.05>

Evaluating Arrowroot Starch Modification and Application in Wet Noodles

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Abstract

Increasing the Resistant Starch (RS) level in food products containing naturally high starch content is important as RS has been acknowledged as a functional food ingredient. The purpose of this study is to determine the effect of steam-cooling treatment on the characteristic of arrowroot starch and to investigate the feasibility of arrowroot-based RS application on wet noodles. The study used a Completely Randomized Design (CRD) with one factor which was the steam-cooling cycle. Later, the substitution of 5%, 10%, and 20% of modified arrowroot starches on wet noodles was used, and then the consumer test was carried out. The results showed that the RS content of arrowroot starch remained after steam-cooling treatment. The treatment, however, reduced the water content and the brightness of the starch. Subsequently, arrowroot starch substitution without or with modification also reduced the lightness and tensile strength of wet noodles. Also, wet noodles with modified arrowroot starch substitution were significantly different in color, taste, and aroma parameters but were still acceptable to the panelists at a maximum substitution of 20%. The formulated wet noodles with modified arrowroot starch contained RS of 34.02% (d/b), and therefore they can be categorized as foodstuffs with high RS levels.

Keywords: arrowroot starch, resistant starch, steam-cooling, wet noodles.

INTRODUCTION

Resistant Starch (RS) is one of the potential ingredients to create functional food. RS is defined as starch that cannot be absorbed in the digestive tract. According to Meenu *et al.* [1], RS can reduce the glycemic and insulin responses in diabetic, hyperinsulinaemic, and dyslipidemic patients. RS reaches the large intestine and thus is fermented by anaerobic bacteria. The fermentation process of RS also has many positive effects on human health, such as increasing the absorption of magnesium and calcium, improving insulin sensitivity, stimulating the immune system, and reducing the risk factors for colon cancer. RS allows a low glycemic response, so it has a low glycemic index (GI), which is useful in slowing down the absorption of glucose into the blood. RS can be classified into four types [2]. RS type I is a natural starch that is physically trapped between the cell walls of foodstuffs. RS type II is a starch granule that is naturally resistant to digestive enzymes. Type III is retrograded starch which is produced through food processing, and type IV is chemically modified starch. RS type III possesses advantages compared to other types of RS as it is stable during heating, and so it can be used as a food

ingredient. Its functional properties remain unchanged during the processing.

Arrowroot starch (*Maranta arundinacea* L) is a potential material processed into a source of RS. Marsono [3] explained the amylose content of arrowroot starch reaches 25.94% per 100 grams of flour. The high amylose content in arrowroot has the potential as an alternative source of producing RS. In the development of commercial RS, it is advisable to use starch which naturally contains high levels of amylose. Moreover, arrowroot is a local food ingredient containing a fairly high economic value and has the potential as an alternative food source. Arrowroot can grow well in humid areas, making it easy to cultivate and produce high yields up to 30 tons.ha⁻¹. Referred to Faridah *et al.* [4], RS in arrowroot starch is type II. Nevertheless, the content of RS type III starch can be enhanced through processing.

It has been widely acknowledged that cooking with the steam method can help increase RS. Sajilata *et al.* [5] mentioned that starch isolated from legumes heated by steam is rich in RS content (19% to 31%, wet weight), while the raw materials did not contain RS. Prolonged steaming and dry pressure heating in a short period may promote the formation of indigestible starch. This formation occurs due to the retrogradation process of starch from cooling, in which retrogradation can change the structure of starch leading to the formation of new insoluble crystals

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that affect the digestibility of starch in the small intestine [6]. Thus, a formation of indigestible starch may occur during steaming-cooling treatment of arrowroot starch, and thus it is hypothesized to significantly increase the RS content of the material.

Formulating RS in popular food is one of the strategies to promote healthy food to the community. Noodles are one of the most widely consumed and popular foods in the community, and thus supplementing RS-rich ingredients is a strategic approach to increase RS consumption in society. From a regulation viewpoint, this strategy is legal, referred to the National Standard of Indonesia released in 1992. Wet noodles can be defined as food products made from flour either with or without the addition of other raw materials and have a maximum water content of 35%.

In this study, the modified arrowroot starch is intended to add into wet noodles, and thus it is hypothesized to increase the level of resistant starch. The purpose of this study, therefore, is to determine the effect of steam-cooling treatment on physical and chemical characteristic of arrowroot starch and also to determine the effect of modified arrowroot starch substitution on physical and chemical properties.

MATERIAL AND METHOD

Preparation of Modified Arrowroot Starch

The production of modified arrowroot starch followed the protocol of Lehmann *et al.* [7]. Arrowroot starch (Ganep's shop, Surakarta, Indonesia) was suspended in a minimum of water and then heated at a temperature of 70°C with continuous stirring. Then it was steamed for 20 minutes. After that, arrowroot starch was cooled down for 1 hour at room temperature, then stored for 24 hours at 4°C. This procedure was repeated for as many variations of the formulation, where F1 = 1 steam-cooling cycle, F2 = 2 steam-cooling cycles, and F3 = 3 steam-cooling cycles. After that, the starch was dried in an oven at 50°C, ground, and sieved.

Production of Wet Noodles

The production of wet noodles followed the protocol published by Bogasari [8]. Wheat flour (Cakra Kembar™ flour, PT. Indofood CBP, Indonesia) was used as the raw material in the making of wet noodles. It was then substituted with arrowroot starch and modified arrowroot starch at a level of 20%. The composite flour was mixed with salt ("Segitiga Emas" salt, PT. Multi

Warna Rasa, Bogor) and soda ash in a bowl. Afterward, water was added gradually and then kneaded until it became a dough. Once it reached the required dough consistency, it was shaped into sheets using a noodle maker machine and then pressed into noodle strands. In the meantime, tapioca flour (Rose Brand™, PT. Budi Acid Jaya, Tbk, Lampung) was subtly sprinkled so that the noodle strands did not stick each other. The finished noodle strands were then boiled in boiling water for 40 seconds. Then, it was drained and dried. Cooked noodles were smeared with cooking oil (Fitri™ cooking oil, PT. Bina Karya Prima Tbk, Tangerang) at about 10 mL per kg of noodles to prevent them from sticking.

Analysis of Resistant Starch Content

The analysis of resistant starch content followed the method of Goni *et al.* [9]. First, 100 mg of the sample was measured into a 25 mL test tube with a screw cap. Then, 10 mL of KCl-HCl buffer (pH 1.5) was added and stirred. Pepsin solution of 0.2 mL (1 g of pepsin. 10 mL⁻¹ of KCl-HCL) was poured and then mixed/shaken for 60 minutes at 40°C. The sample was cooled at room temperature, and then 9 mL of tris maleate buffer (0.1 M, pH 6.9) was added. Amylase solution of 1 mL (40 mg-amylase. mL⁻¹ buffer tris maleate) was poured, then incubated at 37°C for 16 hours with constant shaking. After that, the sample was centrifuged for 15 minutes, as much as 3000 g supernatant was discarded.

The precipitate was added to 10 mL of distilled water and centrifuged, then the supernatant was discarded. The residue was added with 3 mL of distilled water and 3 mL of KOH (4M), and then mixed well and shaken constantly for 30 minutes. HCl of 5.5 mL (2M), 3 mL sodium acetate buffer (0.4 M; pH 4.75), and amyloglucosidase (80 µL, E.c. 3.2.1.3) were then added. The mixture was blended well and incubated at 60°C for 45 minutes and shaken steadily. Later, it was centrifuged for 15 minutes at 3000 g. The supernatant was collected in a 25 mL or 50 mL (S1) volumetric flask. The residue was added with 10 mL of aquadest, mixed, and centrifuged for more. The supernatant (S2) was mixed with the S1 supernatant. Distilled water was added to a certain volume (25 mL or 50 mL) (SA).

Total glucose was determined by the GOD-POD reagent. A standardized glucose solution (10-60 g.mL⁻¹) was used. A total of 1 mL of the GOD-POD reagent solution was added to 20 mL of the supernatant (SA). Then, it was stirred and

incubated in a 37°C water bath for 10 minutes. The absorbance was read at 510 nm. Then, it was compared with blank. RS levels were calculated by Equation 1 and Equation 2:

$$\%RS (wb) = \frac{50}{\text{initial weight}} \times \frac{\text{absorbance}}{100} \times 0.9 \quad \dots\dots\dots 1$$

$$\%RS (db) = RS (wb) \times \frac{100}{(100 - KA (wb))} \quad \dots\dots\dots 2$$

Chemical Analysis

The three variations of the steam-cooling formulation were subjected to physical and chemical analysis. Physical and chemical analyses were carried out on samples of wet noodles with 100% wheat flour, wet noodles with unmodified arrowroot starch substitution, and wet noodles with modified arrowroot starch substitution. The analysis of water, fat, ash, protein and carbohydrate content used thermogravimetric, Soxhlet extraction, dry-ashing, Kjeldahl, and by different methods, respectively, following the standard method of AOAC [10].

Physical Analysis

Color evaluation (Lightness, L*) of the noodles was conducted using a Chromameter (Minolta CR-400, Japan) in CIELAB system. The tensile strength of the noodles was evaluated using Universal Testing Machine (Model Zwick I Z0.5, United Kingdom) according to the ASTM D882-00 standard method [11]. The initial grip distance was set at 50 mm and the crosshead speed was customized at 10 mm.min⁻¹. The tensile strength value was calculated using Equation 3 where F is the force (N) at maximum load and A is the initial cross-sectional area (m²) of the sample. The tensile strength is expressed as MPa.

$$TS = F/A \quad \dots\dots\dots 3$$

Sensory Analysis

In the sensory analysis, 25 panelists were used to evaluate the consumer acceptance of wet noodles. The panelists used in this study are students with a range of age between 18 and 22 years old. A 5-scale evaluation form was used where 1=very much dislike, 2=dislike, 3=neutral, 4=like and 5=very much like [12]. Parameters assessed in this test included color, aroma, taste, texture, and overall.

Data analysis

The research design used a Completely Randomized Design (CRD) with one factor, which was the variation of the steam-cooling cycle as the basis for making highly modified arrowroot

starch RS. One Way ANOVA statistical analysis was administered for the steam-cooling cycle, physical and chemical properties of wet noodles, and sensory results of wet noodles. Then, it was continued with Duncan's analysis for the significance of more than α (0.05) to observe whether there was a difference between treatments. A significance level of >0.01 was particularly used for the statistical analysis of the RS content of arrowroot starch before and after steam-cooling treatment.

RESULT AND DISCUSSION

Steam-Cooling Cycle on Arrowroot Starch

The results of the analysis of RS and color of arrowroot starch are displayed in Table 1. In Table 1, the RS contents of arrowroot starch without treatment in 1 cycle of steam-cooling to 3 cycles were 11.63, 11.76, 10.0, and 11.56%, respectively. Based on the results of the ANOVA test (P<0.01), the modified arrowroot starch did not have a significant difference in all samples shown from the location of the same subset. In other words, the steam-cooling treatment was not able to significantly increase the RS content in the arrowroot starch. It is not in line with the statement by Rosida and Yulistiani [13] that the best treatment is steam-cooling which possibly increased the RS level from 3.27% to 6.67% in breadfruit compared to other treatments, which are boiling-cooling (3.27% to 3.82%) and frying-cooling (3.27% to 3.68%).

Table 1. Resistant Starch content and color of arrowroot starch with and without steam-cooling treatment

Arrowroot Starch	Resistant Starch Content (% db)	Color (L)
Without Treatment	11.63 ^a	93.33 ^d
1 cycle	11.76 ^a	82.54 ^c
2 cycles	10.00 ^a	82.12 ^b
3 cycles	11.56 ^a	80.93 ^a

Note: In the same column, different notations show a significant difference between alpha 0.01 for RS content and alpha 0.05 for color.

The finding in this study did not meet the aforementioned hypothesis. Theoretically, the level of RS in starch-rich material after the steam-cooling treatment increased significantly, mainly due to the amylose content in the starch. Amylose is easily retrograded due to the influence of temperature. The rate of retrogradation will increase as the temperature decreases. The lower the temperature, the faster the retrogradation process will be and the more resistant starch is formed. Cooling after cooking

will change the physical state of polysaccharides, thereby reducing their digestibility [6]. The higher the starch amylose content, the higher the resistant starch content is. Starch granules that are rich in amylose have a greater ability to crystallize due to the higher intensive hydrogen bonding. As a result, starch cannot expand or undergo complete gelatinization during cooking so that it is digested slower.

The disparity may emerge because the starch contained in arrowroot starch is not fully gelatinized during the steaming process. Starch heating with excess water will result in starch gelatinization. Reheating and cooling of the gelatinized starch alters the starch structure, which leads to the formation of new insoluble crystals in the form of retrograded starch [14]. In this study, the incorporation of water was minimal until the arrowroot starch became paste. The use of minimal water content was achieved because during the trial, with excessive water, the gelatinization time will become longer and the gelatinization results will be defective.

Furthermore, as studied by Cham and Suwannaporn [15] using rice flour, hydrothermal treatment (heating) of the flour can suppress swelling and inhibit gelatinization of the granules, so that the starch granules become rigid and more stable during the heating process. The heating treatment can increase the crystallinity region and tend to re-associate to form a precipitate or gel during the drying process (retrogradation). It can also explain the phenomenon in each sample where arrowroot starch was inhibited in gelatinization so that in cycles 1 and 3, arrowroot starch did not have a significant difference in the RS content.

Table 1 also presents the lightness level of arrowroot starch without treatment, 1 cycle to 3 cycles, which were found at 93.33, 82.54, 82.115, and 80.93, respectively. The L value decreased along with the number of steam-cooling cycles in arrowroot starch, implying that the lightness level of arrowroot starch was lower than control. The results of the ANOVA test ($P < 0.05$) presents significant differences in each arrowroot starch sample, denoting that the lightness level of arrowroot starch with different treatments will produce significantly different lightness levels.

Physical and Chemical Properties of Wet Noodles

Based on the results of arrowroot starch modified by the steam-cooling treatment with various cycles, no significant effect on the levels

of resistant starch was found. Nonetheless, it is essential to investigate the physical and chemical features of the product application, which are wet noodles with wheat flour substitution of treated arrowroot starch. The wet noodles made consisted of three types; control wet noodles (100% wheat flour), wet noodles with 20% arrowroot starch substitution, and 20% modified arrowroot starch substitution. Substitution as many as 20% on the previous study opted as acceptable wet noodles to the panelists. The wet noodles were with a ratio of flour 70%, arrowroot flour 20% with the addition of soy flour 10% [16]. In this study, as the emphasis was laid on the effect of arrowroot starch, soy flour was not used. Also, it is widely recognized that the basic recipe of wet noodles is wheat-based flour without any substitution for other types of flour.

The physical characteristics of wet noodles tested in this study included color and texture (tensile strength). Color and texture are the determinants for the quality of wet noodles since they determine the level of consumer acceptance. A Tensile Strength test was performed, which is the maximum force required to break noodles. The greater the force required, the harder the noodles to break. The results of the test of brightness and tensile strength of control wet noodles are wheat flour, wet noodles with arrowroot starch substitution of 20%, and wet noodles with modified arrowroot starch substitution of 20% are provided in Table 2.

Table 2. Physical properties of wet noodles

Formula	Lightness	Tensile Strength (N)
- Control	64.47 ^c	0.18 ^b
- 80% wheat flour + 20% arrowroot starch	55.36 ^a	0.12 ^{ab}
- 80% wheat flour + 20% modified arrowroot starch	60.19 ^b	0.11 ^a

Note: In the same column, different notations indicate a significant difference alpha 0.05

Table 2 shows the lightness level of control wet noodles (100% of wheat flour) was higher than that of wet noodles with 20% arrowroot starch substitution and wet noodles with 20% modified arrowroot starch substitution. It also showed that the lightness levels among samples of wet noodles were significantly different. Interestingly, even though the previous finding shows that modification led to a decrease in the lightness level of arrowroot starch, in this study, the wet noodle formulated with modified starch was lighter than that with unmodified starch.

This phenomenon strongly indicated that many other factors affect the physical properties of wet noodles instead of the color of the raw material.

As for the tensile strength of wet noodles, Table 2 shows a decrease in wet noodles compared to wet noodles with arrowroot starch substitution without treatment and modified arrowroot starch which were 0.18 0.12, and 0.11, respectively. The tensile level of wet noodles decreased due to the substitution of wheat flour with arrowroot starch by 20%, either without treatment or modification. The use of arrowroot starch will reduce the availability of gluten protein, thereby reducing the tensile value of the noodles. Moreover, statistical analysis found that there was a significant difference between control and wet noodles with modified arrowroot starch substitution of 20%. The smaller the gluten protein content in noodles, the lower the elongation ability will be. Gluten protein plays a role in the elasticity of noodles, hence elongation ability decreases [17].

The nutritional compositions of control wheat wet noodles and arrowroot starch substituted wet noodles without treatment and modification are shown in Table 3. It can be seen that the moisture content of wet noodles with modified arrowroot starch substitution was higher than that of control wet noodles, and arrowroot starch substituted wet noodles without treatment. It indicates that the substitution of modified arrowroot starch caused an increase in the moisture content of wet noodles. However, the water content of wet noodles in this study still met the standards set by SNI, where the water content in all wet noodle samples showed less than 35%. The results of the water content imply a significant difference between each sample, which means that either with or without

substitution of modified arrowroot starch, it affected the moisture content of wet noodles.

From the analysis, it was found that the ash content in the control wet noodles was 4.41%, in wet noodles with substitution of unmodified arrowroot starch was 1.06%, and the ash content in wet noodles with substitution of modified arrowroot starch of 20% was 4.45%. The value of the ash content in wet noodles with or without substitution of modified arrowroot starch experienced declining in the reduction in the mineral content of arrowroot starch during the steam-cooling cycle. Statistical results showed that the ash content did not have a significant difference between samples. The ash content qualifies the quality requirements set by SNI at a maximum of 3% (w/b).

The protein contained in wet noodles is generally influenced by the type of wheat flour used and the addition of modified arrowroot starch. The wheat flour used is hard wheat, wheat flour that contains a high protein content of $\pm 12-13$. This type produces dough that has high absorption, is strong, and has good swelling power [17]. Table 3 presents that the protein contents of wet noodles with modified arrowroot starch substitution and ordinary arrowroot starch substitution had a lower content than control wet noodles. It is due to the decrease in gluten content and simultaneously with the decrease in the proportion of wheat flour. In the statistical analysis, significant differences among the samples of control and arrowroot starch substitution without treatment and wet noodles with modified arrowroot starch substitution were found. The protein content of wet noodles in this study qualified the SNI quality standard, at a minimum of 3.

Table 3. Chemical properties of wet noodles

Parameter		Control	Unmodified Arrowroot Starch Substitution	Modified Arrowroot Starch Substitution
Water	%(wb)	54.97 ^b	52.37 ^a	56.8 ^c
Ash	%(wb)	0.57	1.06	0.34
	%(db)	4.41 ^a	4.18 ^a	4.45 ^a
Protein	%(wb)	5.3	4.06	3.77
	%(db)	11.76 ^b	8.76 ^a	8.73 ^a
Fat	%(wb)	2.33	1.79	2.99
	%(db)	5.17 ^b	3.975 ^a	6.65 ^c
Carbohydrate	%(wb)	36.83	39.37	36.1
	%(db)	81.79 ^a	85.07 ^c	83.56 ^b
RS	%(wb)	11.06	15.18	14.70
	%(db)	24.55 ^b	16.59 ^{a*}	34.02 ^c

Note: In the same row, different subsets show a significant difference alpha 0.05 *) at a moisture content of 8.925.

However, indeed, given the nutritional value, noodles contain a lot of carbohydrates and energy with relatively low protein content. The nutritional content of noodles varies, greatly relying on the type, amount, and quality of the ingredients.

In Table 3, the fat content of wet noodles with modified arrowroot starch substitution was surprisingly higher than that of control wet noodles and wet noodles with unmodified arrowroot starch substitution. The substitution was expected to have no significant influence on the fat content of the wet noodles. The difference might be due to the uncontrolled use of cooking oil for smearing the wet noodles to prevent them from sticking.

As for the carbohydrate, wet noodles with substitution of unmodified arrowroot starch had higher content than control wet noodles and wet noodles with modified arrowroot starch substitution, which was 85.07%. It was occurred due to differences in the composition of wheat flour in wet noodles. Wet noodles with modified or unmodified arrowroot starch will contribute more carbohydrates than wet noodles with 100% wheat flour, which contains more protein (gluten). Meanwhile, for resistant starch, wet noodles with modified arrowroot starch substitution had higher resistant starch content than the control, and wet noodles with unmodified arrowroot starch substitution, from 24.55% (db) to 34.02% (db).

These results were obtained because of differences in the composition of wheat flour in wet noodles. Arrowroot starch treated with steam-cooling in the previous analysis resulted in a resistant starch content of 11.76%, which is categorized very high. Goni *et al.* [9] classify foodstuffs based on their resistant starch content in dry weight. Foodstuffs with resistant starch content <1% are classified as very low, 1-2.5% are in the low group, 2.5-5% are in the medium group, 5-15% are in the high class, and >15% are in the very high group. Therefore, due to the very high content of modified arrowroot starch, it will directly affect the wet noodle end product.

Consumer acceptance of wet noodles

Table 4 shows the consumer acceptance of wet noodles formulated with modified arrowroot starch substitution using formulations of 5%, 10%, and 20%. The use of modified arrowroot starch substitution, instead of unmodified arrowroot starch, was based on the fact that modified arrowroot starch substitution in wet noodles resulted in a higher RS content (db) as shown in Table 3, but had a closer lightness level as compared to unmodified arrowroot starch substitution, as shown in Table 2.

Based on the sensory evaluation, the score given by the panelist on the color parameter of wet noodles formulated with modified arrowroot starch substitution of 5%, 10%, and 20% were 3.40, 3.70, and 2.98, respectively. The 10% substitution obtained the highest score of panelist acceptance. The results of the aroma parameter values were 3.03, 3.00, and 3.10, respectively, where all samples of wet noodles had no significant difference. In the taste parameter, consecutive values of 3.10, 3.70, and 3.47 were obtained.

Samples of wet noodles with a formulation of 95% of wheat flour with 5% of modified arrowroot starch and wet noodles of 90% wheat flour with 10% of modified arrowroot starch have a significant difference. Meanwhile, samples of wet noodles with the formulation of 95% of wheat flour with 5% of modified arrowroot starch, wet noodles with 80% wheat flour with 20% modified arrowroot starch, wet noodle with 90% wheat flour with 10% modified arrowroot starch, as well wet noodles with 80% of wheat flour with 20% of modified arrowroot starch were not significantly different. In the texture parameter, the scores of 3.13, 3.47, and 3.67 were obtained, respectively.

Interestingly, the 20% substitution obtained the highest score of panelist acceptance in this attribute. The results of the sensory test in the overall parameter were 3.30, 3.53, 3.57, respectively, and all samples of wet noodles had no significant difference.

Table 4. Consumer acceptance of wet noodles

Flour Formulation (Wheat Flour : Modified Arrowroot Starch)	Color	Aroma	Taste	Texture	Overall
95 : 5	3.40 ^{ab}	3.03 ^a	3.10 ^a	3.13 ^a	3.30 ^a
90 : 10	3.70 ^b	3.00 ^a	3.70 ^b	3.47 ^{ab}	3.53 ^a
80 : 20	2.98 ^a	3.10 ^a	3.47 ^{ab}	3.67 ^b	3.57 ^a

Note: In the same column, different subsets show a significant difference alpha 0.05

Results found in this study are significant for scientists to give a direction for further research, particularly on the process modification for increasing the RS content of modified arrowroot starch. For the food industry, this research shows that substitution of 10% of arrowroot starch in wet noodles obtained the highest score of consumer acceptance, while 20% of arrowroot starch in wet noodles was still acceptable. This information is important for consideration of wet noodle reformulation.

CONCLUSION

To sum up, the steam-cooling treatment had no effect on the resistant starch content of arrowroot starch but significantly affects the decrease in water content and brightness level of arrowroot starch. The more steam-cooling cycles of arrowroot starch, the lower the brightness level of arrowroot starch will be. Modified arrowroot starch substitution had an effect on the physical properties of wet noodles, comprising a decrease in the brightness and tensile strength of wet noodles and had an effect on the chemical properties of wet noodles, which are increasing in water content and RS compared to wet noodles with 100% wheat flour. Variations in the formulation of modified arrowroot starch affected the level of consumer preference on the parameters of color, taste, and texture, in which wet noodles with 20% modified arrowroot starch substitution were accepted by panelists. Given the results of this study, it is suggested to do further research in the form of a combination of chemical modifications or enzymes to increase RS levels and selection of raw materials with high amylose.

Acknowledgement

The authors thank to Universitas Sebelas Maret and the Research Group of Food and Beverage Innovation for financially supporting this publication (Grant No. 2400/UN27.22/PN.01.03/2021).

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Assessing the Genotoxicity Effect of a Commercial Chlorpyrifos Formulation in *Fejervarya limnocharis* Tadpoles (Anura: Dicroglossidae) Under Acute and Chronic Exposure

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Abstract

The potential for genotoxicity of pesticides is currently one of the world's concerns. Chlorpyrifos is the organophosphate active ingredient with the largest sales, but the potential for genotoxicity in amphibians is still not widely known. The purpose of this study was to assess the genotoxicity effect of a commercial chlorpyrifos formulation Dursban 200EC in *F. limnocharis* tadpole erythrocyte (Anura: Dicroglossidae) under acute and chronic exposure using by micronucleus assay. Acute and chronic toxicity tests consisted of negative control, positive control, and 0.4, 0.8, and 1 $\mu\text{g.L}^{-1}$ of chlorpyrifos with three replications. A toxicity test was carried out on ten tadpoles (Gosner 25) from artificial reproductions in each treatment. The results showed that the formulation of Dursban 200EC in low concentrations (0.4 $\mu\text{g.L}^{-1}$) had the potential to induce DNA damage in erythrocytes of *F. limnocharis* tadpoles, and there was a positive correlation between chlorpyrifos concentrations and an increase in the frequency of MN. Erythrocytes exposed to chlorpyrifos in both acute and chronic treatment had significantly different MN frequencies between negative and positive controls, 0.4, 0.8, and 1 $\mu\text{g.L}^{-1}$ ($p < 0.01$). Meanwhile, positive controls were not significantly different from 1 $\mu\text{g.L}^{-1}$ ($p > 0.05$). However, the increase in the frequency of MN in chronic treatment was almost twice as high.

Keywords: Chlorpyrifos, *F. limnocharis*, Genotoxicity, Micronucleus Assay, Tadpoles

INTRODUCTION

The increase in world food demand has led to agricultural expansion, which is often accompanied by excessive and indiscriminate use of pesticides [1,2]. This phenomenon is known to have triggered chemical contamination in the agricultural environment and has been cited as one of the factors causing the decline in local and global amphibian populations [3-6]. Currently, organophosphate (OPs) pesticides are the most widely used pesticides in agriculture [7-9], while these pesticides have relatively no target specifications and are toxic to most non-target organisms, especially amphibians [10,11].

Chlorpyrifos is one of the most active non-systemic organophosphate compounds and is classified as a Class II (moderately toxic) pesticide that is most widely used in agriculture [12,13]. These insecticides act as inhibitors of acetylcholinesterase (AChE), which are involved in neurotransmission in muscles and nerve synapses [14,15], so that they can induce hyperexcitation that triggers paralysis, loss of respiratory control, and even death [10,16]. Chlorpyrifos is known to be toxic to most aquatic organisms [17], while the application of

chlorpyrifos is often done carelessly and excessively so that chlorpyrifos residues are often detected in sediments or water surfaces [18]. Based on the evaluation, the residual chlorpyrifos from runoff, spraying, and spillage in streams and water bodies in the agro-ecosystem ranges from 0.01 to 0.1 mg.L^{-1} [19-21] and in soils ranging from 0.011-0.063 mg.kg^{-1} [9]. In addition, chlorpyrifos has also been shown to induce genotoxicity because it inhibits DNA and protein synthesis at the cellular level [6,22,23].

In contrast to many experimental studies of genotoxicity in rodents and fish, the genotoxicity information of chlorpyrifos in Anura is relatively scarce [24-26], whereas Anura is one of the aquatic organisms that have a high chance of being exposed to chlorpyrifos at all stages of its life [23,27,28]. The micronucleus (MN) test is one of the simplest, fastest, and most sensitive genotoxicity test methods, especially for aquatic organisms and amphibians [24,29]. The results of the evaluation of chlorpyrifos toxicity in Anura showed different and species-specific results [14, 15,23,30]. It indicates that additional studies on other species need to be carried out [14].

Fejervarya limnocharis (Anura: Dicroglossidae) is one of the Anura species that has great potential for exposure to chlorpyrifos, especially in the tadpole phase because it lives and breeds in shallow waters of the agroecosystems and is widespread in Asia where there are intensive agricultural activities [31-34].

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Therefore, the assessment of the genotoxicity effect of chlorpyrifos on *F. limnocharis* tadpoles is important to know because DNA damage from genotoxic stress can threaten the integrity of the organism [35], while the species is an integral component of the agroecosystems [34,36].

This study was aimed to assess the genotoxicity effect of a commercial chlorpyrifos formulation in *Fejervarya limnocharis* tadpole erythrocyte (Anura: Dicroglossidae) under acute and chronic exposure by using Micronucleus assay. This study was expected to be supporting data to determine the level of toxicity of chlorpyrifos to Anura.

MATERIAL AND METHOD

Ethical Clearance

All experiments involving animals were performed following ethical standards. The University of Brawijaya ethics committee evaluated and approved the study protocol with the registration number of ethical clearance, 092-KEP-UB-2021.

Experimental Design

This study consisted of acute and chronic toxicity tests with working procedures adapted from literature [21,37-40]. The treatments in both tests consisted of control (NC and PC), 0.4, 0.8, and 1 $\mu\text{g.L}^{-1}$ chlorpyrifos with three replications at each concentration. The negative control (NC) consisted of tadpoles that were reared in dechlorinated water without any mixture, while the positive control (PC) used 5 mg.L^{-1} cyclophosphamide (CF). The treatments were carried out on ten pooled random tadpoles (Gosner stage 25) for each repetition. The Gosner stage is a generalized system to describe the stages of embryonic and larva development in anurans [41]. A toxicity test was carried out on the test solution with a volume of 3L with natural light and dark cycle for each treatment. The acute toxicity test was carried out for 96 hours of treatment, while the chronic toxicity test was carried out until there were tadpoles in NC entering Gosner 46. The parameter evaluated in this study was the number of micronucleated erythrocytes from each treatment. In each treatment, five tadpoles were taken as evaluation samples, so that five preparations were obtained for each treatment.

Treatment Preparation

Test Organism

The *F. limnocharis* tadpoles were obtained from artificial reproduction using a method that referred to the research of Kurniawan *et al.* [42].

Adult *F. limnocharis* were collected from rice fields in Blayu Village, Wajak, Malang Regency, East Java, Indonesia (Loc. 8°06'55.62" S 112°44'23.87" E). The process of acclimatization to semi-natural habitats and artificial reproduction is carried out in the Laboratory of Ecology and Animal Diversity, University of Brawijaya.

The gonadal maturation process in female individuals was initiated by injecting the Trial Batch of 2000 IU hCG by Kings Lab at a dose of 1 mL.kg^{-1} dissolved in 0.9% physiological NaCl. The injection is done intraperitoneally, and after 8-10 hours, the eggs are removed by the stripping method. Fertilization was carried out for 10-15 minutes by mixing eggs and sperm suspension from the crushed male frog testes. The fertilized eggs were then transferred to a plastic container measuring 53 × 38 × 17 cm containing 10 L of dechlorinated water equipped with aeration at a water temperature of 20-25°C. The egg mass was allowed to hatch, and two days after hatching, the tadpoles were given feed in the form of boiled spinach and boiled egg yolk that had been mashed. Tadpoles that have reached Gosner stage 25 [41] are then randomly selected from the rearing tank as test animals.

Test Solution

Commercial grade chlorpyrifos with the trademark Dursban 200 EC containing 200 g.L^{-1} chlorpyrifos (PT Dow AgroSciences, Indonesia, RI. 01010119746) was purchased from a local pesticide dealer. Stock solutions with a concentration of 1 mL.L^{-1} were prepared using dechlorinated tap water, while test solutions based on effective concentrations for acute and chronic toxicity tests were prepared by diluting the stock solution. The test solution for the positive control was prepared to dissolve 5 mg CF per one liter of dechlorinated water.

Treatment

Acute Toxicity Bioassay

An acute toxicity bioassay on *F. limnocharis* tadpoles was carried out for 96 hours. Five treatments were tested, consisting of negative control (NC), positive control (PC), and three levels of chlorpyrifos concentration (0.4, 0.8, and 1 $\mu\text{g.L}^{-1}$). NC consisted of tadpoles reared in dechlorinated water, while PC consisted of tadpoles reared in 5 mg.L^{-1} CF solution. The treatment for each concentration and control was carried out on ten Gosner 25 tadpoles [35] with three replications. Samples of tadpoles that have been randomly selected are then

transferred to a plastic container with 3 L of the test solution. During the experiment, the experimental animals were kept under controlled water conditions with a temperature of $24 \pm 2^\circ\text{C}$, a median 7.1 pH, and a natural light/dark cycle.

Chronic Toxicity

The chronic toxicity test in this study consisted of five treatments which were divided into negative control (NC), positive control (PC), and three levels of chlorpyrifos concentration (0.4, 0.8, and $1 \mu\text{g.L}^{-1}$). The test concentrations are obtained from the result of the preliminary test. The treatment was carried out in a plastic container containing 3 L of the test solution with ten Gosner 25 tadpoles [41] with three replications for each concentration. The period of chronic exposure was carried out from Gosner 25 to Gosner 46 [41].

During the experiment, all animals were kept under controlled water conditions with a temperature of $24 \pm 2^\circ\text{C}$, a median pH of 7.1, and a natural light/dark cycle. Update of the test solution was carried out every nine days. The tadpoles were fed boiled organic spinach and boiled egg yolks that had been mashed three times a week during the exposure period up to Gosner 41. Tadpoles that already had forelegs (Gosner 42) were kept in tanks with an inclination of 10° so that there was a dry area. During this period, the tadpoles were not fed until the tail is completely absorbed because the tadpoles have obtained nutrition from the fat stored in the tail.

Data Collection

Micronucleus Assay (MN)

F. limnocharis tadpoles for each acute and chronic treatment were prepared in duplicate. At the end of exposure, a blood smear is made of

the blood removed from the heart for each tadpole. The blood sample was placed on a glass slide and then smeared. Next, it was fixed with absolute methanol for 10 minutes, air-dried, and stained with Giemsa 6% for 20 minutes. Five smear preparations from each acute and chronic treatment were then observed using a light microscope (Olympus, Type CX21LEDFS1-1-2, oil immersion lens, 100/1.25) to determine the frequency of micronuclear cells (MN%). The number of count cells is 1000 cells from five fields of view. The calculation results obtained are then calculated as cells per 1000 (‰) with the following formula [24]:

$$\text{MN } \text{‰} = \frac{\text{number of micronucleus}}{\text{total number of cell counted}} \times 1000$$

Data Analysis

The data were statically analyzed using the SPSS program with One Way of Variance Analysis (ANOVA) and were continued to the Post Hoc test with the p-value < 0.05 .

RESULT AND DISCUSSION

Erythrocytes of *F. limnocharis* Tadpole

The mature erythrocytes of the *F. limnocharis* tadpoles were elongated oval in shape with the structure and position of the nucleus visible so that the presence or absence of fragments in the cytoplasm can be easily identified. Micronuclei (MN) induced by commercial formulations of chlorpyrifos in erythrocyte cells were generally dot-shaped separated from the main nucleus with shapes and sizes that vary between cells. Most of the erythrocytes exposed to chlorpyrifos had a single micronucleus (Fig. 1A) while some cells had more than one micronucleus (Fig. B) and some had larger micronuclei sizes.

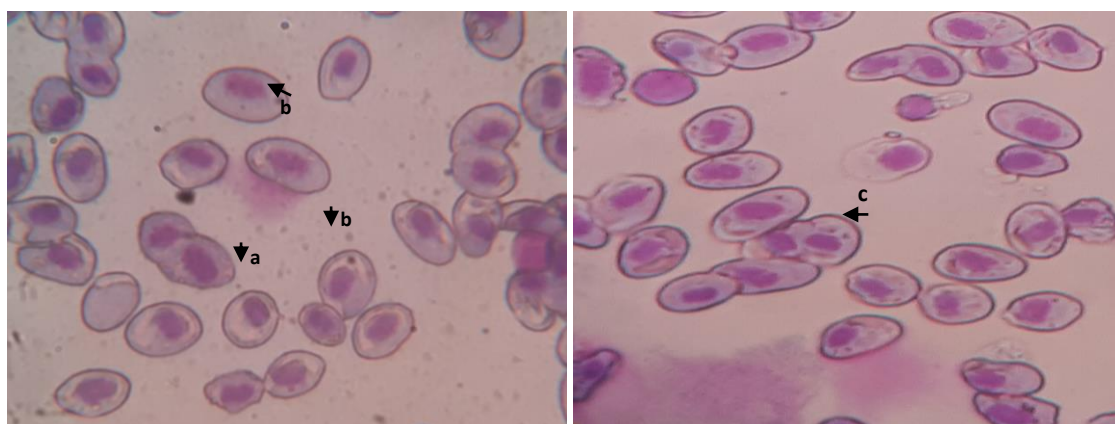


Figure 1. Erythrocytes of *F. limnocharis* tadpole exposed to the commercial organophosphate insecticide Chlorpyrifos ($1 \mu\text{g.L}^{-1}$). Blood smear stained with Giemsa, 1000x . a) Normal erythrocytes, b) Single micronucleus, c) Double micronucleus.

Micronucleus (MN) analysis is widely used as a method to evaluate the genotoxicity effect of pollutants on several organisms, especially amphibians, by detecting the presence or absence of small chromosomal fragments produced by clastogens or chromosomes [24-26]. The formation of MN in amphibian tadpole erythrocytes that were exposed to pesticides has been well demonstrated in previous studies [43,44], while the micronucleus was an indicator of genetic damage [24,25]. The results of research by Bhatnagar *et al.* [45] stated that exposure to chlorpyrifos was able to induce changes in cell morphology, the presence of nuclear anomalies such as damage to the nucleus, and the presence of large micronuclei. In the study of Yin *et al.* [25], exposure to chlorpyrifos of the tadpoles *Bufo bufo gargarizans* showed the presence of nuclear pyknosis, two-nucleated erythrocytes, and changes in erythrocyte cell morphology.

Micronucleus Frequency

Acute Exposure

The results of the genotoxicity test from acute exposure showed the potential for low doses of commercial chlorpyrifos formulations to induce DNA damage in erythrocyte cells of *F. limnocharis* tadpoles and a positive correlation between chlorpyrifos concentrations and increased micronuclear frequency (MN%). MN frequency of all treatment groups consisting of positive control ($57.2 \pm 3.8\%$), $0.4 \mu\text{g.L}^{-1}$ ($40.8 \pm 3.7\%$), $0.8 \mu\text{g.L}^{-1}$ ($48.8 \pm 2.3\%$), and $1 \mu\text{g.L}^{-1}$ ($59.6 \pm 8.8\%$) was significantly different ($p < 0.01$) with the negative control group ($18 \pm 4.9\%$). Meanwhile, the increase in the frequency of MN in the positive control was not significantly different by $1 \mu\text{g.L}^{-1}$ ($p > 0.05$) (Fig. 2).

This study showed that acute exposure to a commercial formulation of chlorpyrifos in low concentrations ($0.4 \mu\text{g.L}^{-1}$) induced DNA damage in erythrocytes of *F. limnocharis* tadpoles. Chlorpyrifos exposure to *Bufo bufo gargarizans* tadpoles with a concentration of 0.16; 0.32 and 0.64 mg.L^{-1} [25] and 10, 100, 200, and 400 mg.L^{-1} in the tadpole *O. carvalhoi* [13] for 96 hours showed a significant increase in micronuclear frequency after being compared with negative controls [13,24,25]. These results indicated that *F. limnocharis* tadpoles had a higher sensitivity to chlorpyrifos than the results of previous studies on *B. b. gargarizans* and *O. carvalhoi* tadpoles.

This variation is most likely caused by differences in the resistance of the test species

[25]. The difference in sensitivity of commercial chlorpyrifos between these species confirms the idea of a species-specific possibility. Thus, the tadpoles of *F. limnocharis* species may be more sensitive to the toxic effects of chlorpyrifos in lower concentrations when compared to other species in the literature.

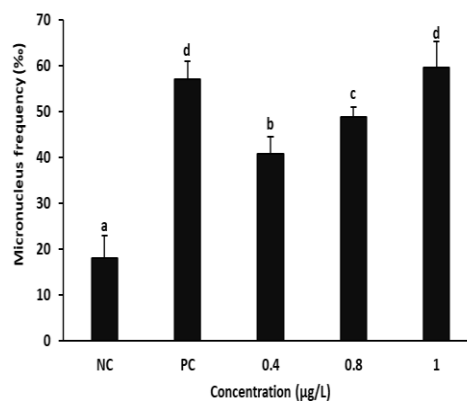


Figure 2. Micronucleus frequency (%) of *F. limnocharis* tadpoles exposed to different chlorpyrifos concentrations under acute exposure. The a, b, c and d letters showed a significant difference compared to the control group (NC) ($P < 0.05$) according to the ANOVA test at each concentration

Chronic Exposure

Long-term exposure of the commercial formulation of chlorpyrifos showed the potential to induce DNA damage with the formation of micronuclei in erythrocytes of *F. limnocharis* tadpoles after 36 days of exposure. The results of the genotoxicity test showed a positive correlation between the concentration of chlorpyrifos and an increase in the frequency of micronuclei (MN). The MN frequency of individuals in the negative control group ($32.8 \pm 6.6\%$) had significantly different results with the positive control ($119 \pm 9.6\%$), $0.4 \mu\text{g.L}^{-1}$ ($92.8 \pm 5.2\%$), $0.8 \mu\text{g.L}^{-1}$ ($113 \pm 6.4\%$) and $1 \mu\text{g.L}^{-1}$ ($121 \pm 8.1\%$) ($p < 0.01$). The increase in the frequency of MN in the positive control was not significantly different from the $1 \mu\text{g.L}^{-1}$ treatment ($p > 0.05$) (Fig. 3).

The results of long-term exposure with the same concentration as acute exposure showed an almost twofold increase in the frequency of MN compared to the results of acute exposure. All concentrations of chlorpyrifos assay can induce much higher micronucleus counts compared to controls [44]. Yin *et al.* [25] reported that exposure to chlorpyrifos in Anura could significantly increase the frequency of MN in proportion to the increase in concentration

and duration of exposure. Similar results have been reported by several other studies in humans [45], fish [46], and tadpoles [47].

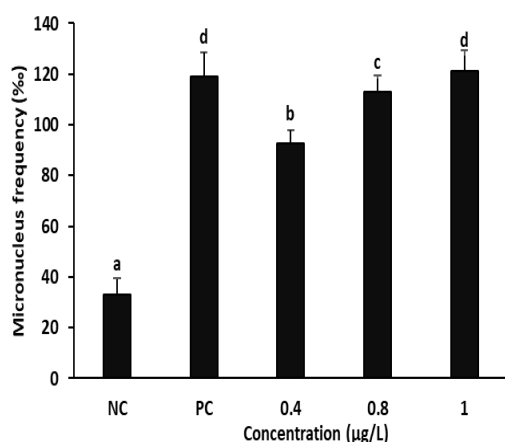


Figure 3. Micronucleus frequency (%) of *F. limnocharis* tadpoles exposed to different chlorpyrifos concentrations under chronic exposure. The a, b, c and d letters a significant difference compared to the control group (NC) ($P < 0.05$) according to the ANOVA test at each concentration.

In summary, the commercial formulation of chlorpyrifos (Dursban 200EC) could cause DNA damage in erythrocyte cells of *F. Limnocharis* tadpoles. The mechanism of DNA damage triggered by chlorpyrifos can be through oxidative stress mechanisms or the mitochondrial pathway [48-50]. MN and morphological changes of the erythrocyte nucleus can trigger genetic mutations, metabolic damage, pathology, and pathophysiological conditions so that it can affect or reduce physical conditions, species survival, and loss of local diversity. *O. carvalhoi* tadpoles were susceptible to genetic changes caused by the use of the commercial formula chlorpyrifos (Klorpan®) [13]. Thus, the results of this study further show that chlorpyrifos can induce micronucleus and DNA damage in tadpole erythrocytes. Therefore, it is possible that the results could support further genotoxic studies in other Anura species and for the understanding of sustainable agricultural models that do not compromise the sustainability of amphibian communities.

CONCLUSION

The genotoxicity assessment showed that the commercial chlorpyrifos formulation of Dursban 200EC had potential genotoxicity in *F. limnocharis* tadpoles. It is known that it can induce an increase in the formation of micronuclei, even at the lowest concentration

(0.4 µg.L⁻¹). There was a positive correlation between the concentration of chlorpyrifos and an increase in the frequency of micronuclei (MN) in both the short-term and long-term treatment. As this is the first study to examine the genotoxicity effect of the chlorpyrifos on *F. limnocharis* tadpoles, it establishes baseline data that can be used as general reference values for future investigations involving this species and other anuran tadpoles.

ACKNOWLEDGEMENT

The researcher would like to thank all NK Research members. This research is supported by the DIPA project of the University of Brawijaya 2021 (DIPA-042.01.2.400919/ (2021)).

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Phytochemical Analysis of Purple Sweet Potatoes (*Ipomoea batatas*) Roots Extract From Lawang and Kawi Mountain Cultivar, East Java, Indonesia

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Abstract

Indonesia has wide cultivation of purple sweet potatoes (PSP) commodities, particularly in East Java province. However, the difference of phytochemical profiles in PSP among geographical regions has not been fully explored. This study aimed to analyze the phytochemicals, anthocyanin, and antioxidant activity profiles from two different cultivars of PSP from Lawang and Kawi Mountain region, East Java, Indonesia. The acidified methanol extract was identified for a phytochemical compound using standard methods. Antioxidant activity was analyzed using a ferric reducing assay. Anthocyanins were screened using ultraviolet-visible spectroscopy and total calculation. Both extracts have positive values in their alkaloid, phenolic, flavonoid, glycoside, and tannin content. Antioxidant activity was high with IC₅₀ value 2.5 and 2.3 µg. mL⁻¹ for Lawang and Kawi Mountain, respectively. Each cultivar has a similar peak at 521 nm at pH 1 and 530 nm at pH 4.5. Total anthocyanin calculation was showed that Lawang has higher anthocyanin content than Kawi Mountain cultivar. We concluded that PSP from Lawang has better anthocyanin content than the Kawi Mountain cultivar. We proposed that PSP from the Lawang cultivar has the potential to be explored in further research and health-related product development.

Keywords: anthocyanin, antioxidant, geographical, purple sweet potatoes.

INTRODUCTION

Sweet potatoes (*Ipomoea batatas* (L) Lam) is an important annual perennial plant that contributed as global food security [1]. Indonesia is one of the major supplier of sweet potatoes commodity worldwide. The biodiversity of sweet potatoes in Indonesia is addressed into natural resources that should be taken into consideration, especially purple sweet potatoes varieties [2,3]. Purple sweet potatoes (PSP) cultivation is dominant in East Java Province, particularly in Malang District at Lawang and Kawi Mountain region [4]. The attractive color pigment and higher anthocyanin compound are more favorable than another phenotypes. Purple sweet potatoes are the main source of energy, amino acid, macronutrient, and anthocyanin. Anthocyanin in PSP previously reported has beneficial biological activity on inflammation, cancer, mutation, oxidative stress, liver protection, etc. [5,6].

Purple sweet potatoes from the Kawi Mountain region displayed potential health benefits in inhibiting the brain cell apoptosis in an animal model of diabetes as well as enhancing the antioxidant enzyme in atherogenic rats [7,8]. However, research on the nutritional content of

PSP from Kawi Mountain cultivar is limited to amino acid characterization [9]. Meanwhile, nutritional identification of PSP cultivated in Lawang is limited to a major proximate composition, for instance, protein, carbohydrates, fat, water content, and ash [10].

The growth environment is strongly correlated with the synthesise of plant bioactive compounds [11]. The validation of plant growth conditions is important in the functional food development process to ensure product quality and safety [12]. Thus, the profiling of biochemical compounds of PSP from Lawang and Kawi Mountain is necessary to support the potential of both cultivars to be developed into functional food. This research was evaluated the differences of phytochemicals, anthocyanins, and antioxidant activity of PSP from those different geographical. A recent study provides the recommendation of the utilization of both cultivars according to their biochemical compounds.

MATERIAL AND METHOD

Plants extraction

Tuber roots of purple sweet potatoes (PSP) cultivar of Lawang and Kawi Mountain were obtained from the local traditional farmer and immediately transfer to the laboratory. Solid extraction was performed according to a previous protocol with minor modification. In brief, the procedure was performed by grinding the fresh tuber roots (50 g) followed by

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maceration in 100 mL acidified methanol 1% (CAS No. 67-56-1, Merck) for 24 hours at 25°C. The homogenates were filtrated using Whatman paper 0.45 µm. Repeated maceration and filtration were conducted until colorless extract was obtained. The filtrates were evaporated at 50°C using a rotary evaporator RE-25C 1L series. The filtrates were then stored at a temperature of 4°C until used [13].

Qualitative phytochemical screening

A standard method as previously described was performed to evaluate the alkaloid, phenolics, flavonoid, glycosides, and tannin. The absorbance of alkaloid, phenol, flavonoid, glycoside, and tannin was measured at 470, 280, 430, 210, and 700 nm wavelength, respectively [13].

Ultraviolet-visible spectroscopy

Anthocyanin preliminary structure determination was conducted by measuring the absorbance with UV-1700-spectrophotometer (Shimadzu, Japan) at pH 1 and pH 4.5 conditions. The maximum absorbance in acidic solution was used for indicating the basic structure of anthocyanins. A ratio of 1:100 (v/v) PSP extract and buffer solution was used for absorbance measurement. A total 1 mL PSP extract was dissolved with 0.25 M HCl-KCl (CAS No. 7447-40-7, Merck) buffer (pH 1) or 0.5 M sodium acetate buffer (pH 4.5) [6,13,14].

Anthocyanin calculation

Total anthocyanin content (TAC) was expressed as cyanidin-3-glucoside per 100 g (C3Gmg.100g⁻¹). The calculation of TAC was conducted using the following formula [15]:

$$TAC = \left(\frac{A}{\epsilon \times L} \right) \times MW \times DF \times \left(\frac{V}{Wt} \right) \times 100$$

Description:

TAC = Total Anthocyanin Content

A = Absorbance

MW = molecular weight (449.2 gmol⁻¹)

DF = dilution factor

ε = molar extinction coefficient (26.900 molcm⁻¹)

L = cuvette diameter

V = final volume (L)

Wt = extract weight (g)

Antioxidant activity

The reducing power assay method was performed to measure the antioxidant activity of both extracts with minor modification. A series of extract concentration of 0, 2, 4, 6, 8, and 10 µg.mL⁻¹ was made by dilution of 2.5 mL, pH 6.6 pH phosphate buffer and 2.5 mL, 1% potassium

ferricyanide. The mixture was incubated in a water bath at 50°C for 30 minutes with a slightly shaking movement. Subsequently, a total of 2.5 mL 10% trichloroacetic acid was added to the mixture. The mixture (5 mL) was dissolved with aquadest (5 mL) and 0.1% FeCl₃ (1 mL). The absorbance of the reaction mixtures was measured at 700 nm. Ascorbic acid was used as a positive control [16]. The IC₅₀ value was calculated as previous report [17].

Statistical analysis

The differences of total anthocyanin purple sweet potatoes Lawang and Kawi mountain were analyzed using an independent-sample of t-test by SPSS software. Meanwhile, the IC₅₀ difference between ascorbic acid of Lawang and Kawi Mountain cultivar was analyzed using one-way ANOVA. Data were shown as mean ± SD from three replication. The level of significance was set as p<0.05.

RESULT AND DISCUSSION

Phytochemical Profiles of Purple Sweet Potatoes From Lawang and Kawi Mountain.

Qualitative phytochemical screening was revealed the presence of alkaloid, phenolic, flavonoid, glycoside, and tannin in both extracts of PSP. However, the cultivar of Lawang was a relatively low color intensity for alkaloid and higher color intensity for flavonoid (Fig. 1).

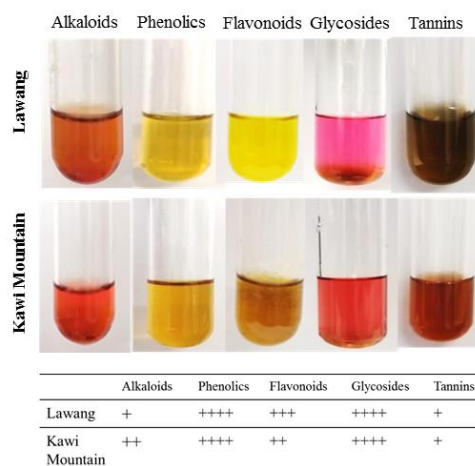


Figure 1. Qualitative phytochemical profiles from extracts of purple sweet potatoes Lawang and Kawi Mountain cultivars. The presence of tested compounds was visualized as + low, ++ medium, +++ high and ++++ very high intensity color

The present study was revealed that both cultivars have similar phytochemical content. Phytochemical content in sweet potatoes can be affected by several factors, for instance, the

genetic color of tuber roots. The purple and red color of tuber flesh were reported higher flavonoid and phenolic content than other white flesh [18]. The application of plant growth regulators has increased the quantitative phytochemical matters in sweet potatoes [19]. A previous study was suggested that phytochemical compounds such as alkaloids, glycosides, tannins, flavonoids, and phenolics in PSP are related to antioxidant activity [20].

Antioxidant activity of Purple Sweet Potatoes From Lawang and Kawi Mountain.

Antioxidant activity and IC₅₀ value are negative correlations. Both PSP cultivars were shown antioxidant activity was directly proportional to the increase in concentration. PSP Lawang cultivar was not significantly different from PSP Kawi Mountain cultivar at the IC₅₀ value, i.e 2.5, and 2.3 µg.mL⁻¹ for Lawang and Kawi Mountain, respectively (Fig. 2). Thereupon, the antioxidant activity of the Lawang cultivar was relatively similar to Kawi Mountain cultivar.

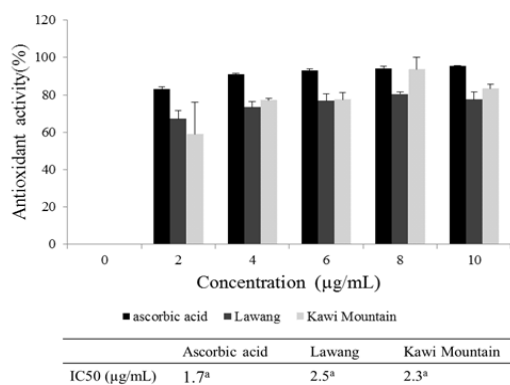


Figure 2. Similar antioxidant activity is observed from the value of IC₅₀. The same notation is reflected no significant value ($p=0.076$). Data were shown as mean value \pm SEM.

The higher antioxidant activity is also influenced by the presence of phenolics and flavonoids in purple sweet potatoes [21]. In addition, purple sweet potatoes have higher levels of anthocyanin as a powerful antioxidant [22].

Anthocyanin in Purple Sweet Potatoes From Lawang and Kawi Mountain.

The UV-visible spectroscopy analysis was demonstrated that both extracts were absorbed UV light at a range of 200-324 nm and visible light at a range of 469-572 nm under pH 1 condition. Both cultivars have the same peak of absorbance at 521 nm in pH 1 and 530 nm in pH 4.5 (Fig. 3A and 3B). The range of peak between 520-530 nm is characterized as anthocyanin

absorption wavelength. A similar study by Li *et al.* reported maximum absorption from purple sweet potato extracts at 520-530 nm using UV-vis spectrophotometer analysis [23].

The similar range of hump and peak on UV-spectroscopy indicated that both cultivars have a similar basic structure of anthocyanin. The peak of absorbance under the UV-light at 521 nm and the acid condition was correlated to anthocyanin pigment content prediction in plants. Basic structures of anthocyanin in both cultivars are predicted as acetylated anthocyanin as previously reviewed. Acetylated anthocyanin is formed by sugar attachment on anthocyanidin moiety [6,24].

Even though both cultivars have similar wavelength hump peaks of absorbance, the 521 nm absorbance was higher in Lawang cultivar than the Kawi Mountain cultivar. It correlates with the higher total anthocyanin content in Lawang cultivar than Kawi Mountain cultivar, i.e., 10 mg.100g⁻¹ and 5 mg.100g⁻¹ respectively, with $p=0.000$ (Fig. 3C). The flesh color of various potatoes previously was correlated with different total anthocyanin content. It is supported by Teow *et al.*, which displayed a not detected level of anthocyanin in white and orange sweet potatoes. Despite the different anthocyanin content among cultivars, the antioxidant activity was similar ($p=0.076$). Phytochemical composition affects the antioxidant activity in plants, such as phenol, phenols, ortho-diphenols, flavonoids, and tannins [25].

Environmental factors such as the difference in growth altitude, soil pH, daily temperature, and light intensity were contributed to the content of phytochemical compounds in plants [26]. The range of daily temperature has affected the accumulation of anthocyanin biosynthesis. The increase of night temperature from 12-22°C and day temperature to 25°C promotes the increase of anthocyanin as well as radical absorbance capacity [27]. Soil characteristics were contributed to anthocyanin synthesis in plants [28,29]. Lawang and Kawi Mountain region has distinct environmental characteristics such as temperature and soil pH. The Lawang region has a daily temperature of 4°C lower than the Kawi Mountain region. The soil pH of the Kawi Mountain region is more acid than Lawang i.e., 4.71 and 5.25, respectively.

The previous review was reported that the accumulation of anthocyanin is affected by water drought and salt stress through cytoplasm dehydration [11]. However, the potatoes plant

was shown distinct responses towards drought stress. Drought has resulted in the decrease of tuber yield without a significant difference in anthocyanin and antioxidant measurement. Nevertheless, simultaneous application of drought and wound to purple potatoes

significantly reduce the Trolox equivalent. It suggested that purple potatoes are more sensitive to drought, especially during tuber bulking, with a less pronounced effect on anthocyanin production [30].

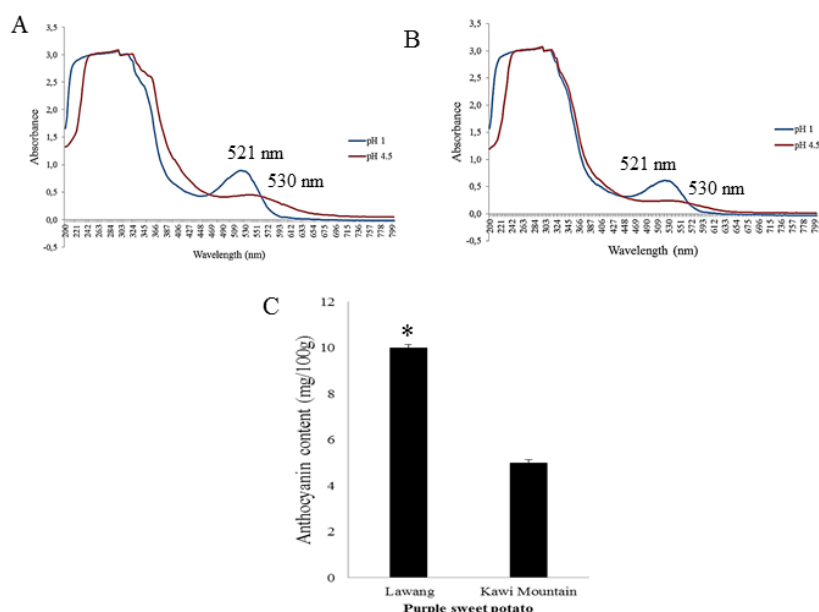


Figure 3. Anthocyanin contents from two cultivars of purple sweet potatoes. A) The absorbance of UV-Vis spectroscopy profiles of Lawang cultivar and B) Kawi Mountain cultivar. Despite both cultivars have similar peak at 521 nm wavelength under pH 1 (blue line) and shifted to 530 nm in pH 4.5 (red line), the absorbance level of Lawang Cultivar at 521 nm is higher than Kawi Mountain cultivar C). The total anthocyanin of Lawang cultivar significantly higher than Kawi Mountain cultivar with $p=0.000$. The (*) notation identified significantly different. The data were shown mean value \pm SEM.

CONCLUSION

Purple sweet potatoes from Lawang and Kawi Mountain region of East Java Indonesia have similar qualitative phytochemical profiles and antioxidant activity. However, anthocyanin content in Lawang cultivar was higher than Kawi Mountain cultivar. We proposed the necessary study related to environmental characteristic profile in both regions to support the growth requirements enrichment to enhance the anthocyanin as well as other secondary metabolites in both cultivars.

Acknowledgements

This research was partially funded by Research and Community Services Agencies, Faculty of Medicine, Universitas Brawijaya, no. 214.26/SK/UN10.F08.06/PN/2020. We thank all members of Smart Molecule of Natural Genetics Resource Research Centre Universitas Brawijaya.

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Analysis of Microplastics in Water and Biofilm Matrices in Metro River, East Java, Indonesia

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Abstract

The Metro River flows from upstream to downstream across East Java to support several human activities such as household, toilets, or agriculture. The utilization of water rivers must be balanced with water quality monitoring so that the quality of the water can be monitored. This study aims to analyze the abundance of microplastic in the Metro River. Moreover, the water parameters (pH, dissolved oxygen, temperature, flow velocity) were also measured. Sampling was carried out at three different stations, namely station 1 representing the agricultural activity area, station 2 representing the household activity area, and station 3 representing industrial activity. This study shows the abundance of microplastics in biofilm matrices and surrounding river water. The types of the microplastics are fibers, fragments, and films. The total abundances of microplastics in river water ranged from 0.8 - 1.61 particle.mL⁻¹, while the biofilm matrices ranged from 7.4 to 9.5 particle.gram⁻¹. The results of water quality parameters at all stations are still relatively good compared to quality standards. To the best of our knowledge, this study was the first study that reports the microplastics in the water of and inside biofilm formed on Metro River.

Keywords: aquatic ecology, biofilm, Metro River, microplastics, water pollution.

INTRODUCTION

Economic growth and an increase in population, primarily in big cities, can harm the environment. Environmental pollution can occur everywhere, including in the aquatic ecosystem. If directly discharged into the waters, various wastes from human activities such as households, industry, and agriculture can increase the concentration of pollutants and reduce the quality of the waters [1]. Meanwhile, water has a crucial role in the survival of humans, animals, and plants. Therefore, this problem requires proper handling.

Open and flowing waters such as rivers have the potential to be polluted. River pollution may occur due to waste from various human activities. The presence of pollutants will change the river's physical, chemical, and biological conditions [2]. Solid waste or garbage is the primary pollutant material intentionally or unintentionally carried by runoff water into the watershed. One of the most common types of waste is plastic waste which can be degraded into microplastics. Although it has been seen as one of the substances that can become emerging pollutants, studies on microplastics in rivers in

Indonesia are rarely reported. Nevertheless, studies on the content of microplastics in river ecosystems in Indonesia are an essential part of managing river ecosystems [3-6].

Microplastic is plastic waste that has a size of less than 5 mm. The change of plastic waste into microplastics takes a long time through the degradation process. Therefore, microplastics in waters are a severe problem because of their persistence, harming aquatic biota. Microplastics have various shapes, colors, sizes and are widely distributed in waters worldwide [7]. Microplastic pollution cannot be easily removed from the waters because of its persistent nature. The level of contamination of microplastic pollution can impact the food chain in marine waters, ranging from microorganisms such as plankton, various species of fish, and marine mammals [8]. The use of rivers for various human activities allows the presence of contaminants in the form of microplastics. However, research is rarely done to analyze the abundance of microplastics in river ecosystems.

Microplastics can be adsorbed into biofilms which are the dominant habitat of microbes in aquatic ecosystems. Microplastics in biofilms can be a problem in aquatic ecosystems because biofilms are actively involved in the food chain process. In this food chain process, microplastics in the biofilm can move and accumulate in fish that eat the biofilm. If humans consume these

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fish, these microplastics can be accumulated in the human body.

The Metro River is a subsidiary of the Brantas Watershed, which flows throughout Malang City and ends in Kepanjen District, Malang Regency, with a river length of 54.55 km. This river has many benefits to support various human activities, especially for residential residents in watersheds. Based on its designation, the Metro River is a class II water class. It means water designation can be used for water recreation infrastructure/facilities, freshwater fish cultivation, animal husbandry, water for irrigating crops, or others that require the same water quality as that use [9].

This study aims to analyze the content of microplastics in water and biofilms growing in the Metro River. This study provides essential knowledge to develop bioassessment technology for aquatic environment management. This study was the first that reports the microplastics in the water and biofilm formed on Metro River.

MATERIAL AND METHOD

Sampling Area

This research was conducted in the Metro River, Malang City, in September 2021. Water and biofilm sampling was carried out at three different stations, each station representing an area of agricultural, household, and industrial activity. The sampling method used in this research is purposive sampling. A sampling at each station was also repeated three times. The

sampling location of this research can be seen in Figure 1.

Station 1 is the Metro River located near Tirtomulyo Street, Tlogomas Village, Lowokwaru District. The coordinates of station 1 for sampling this research are 7°56'17" S and 112°35'51" E. This location is a watershed affected by waste from household and agricultural activities.

Station 2 is the Metro River located on Klayatan Street, Bandungrejosari Village, Sukun District. The coordinates of 7°59'58" S and 112°37'04" E were for Station 2 sampling. This watershed is affected by waste from household activities. Station 3 is the Metro River located at Raya Kebonagung Street, Karang Sono Village, Pakisaji District. The coordinates of station 3 are 8°02'13" S and 112°36'41" E. This watershed area is affected by waste from household activities and several industrial activities.

Sampling Procedure

The water parameters measured in this study were pH, dissolved oxygen, temperature, and water flow velocity. Measurement of physical and chemical water quality parameters was carried out in situ. Temperature (°C) was measured using a digital thermometer. The pH was measured using a pH meter (Lutron PH-201), the water flow velocity (m.s^{-1}) was measured using a current meter (JDC Flowatch FL-03). The dissolved oxygen (ppm) was measured using a DO meter (Lutron DO-5509) [10].

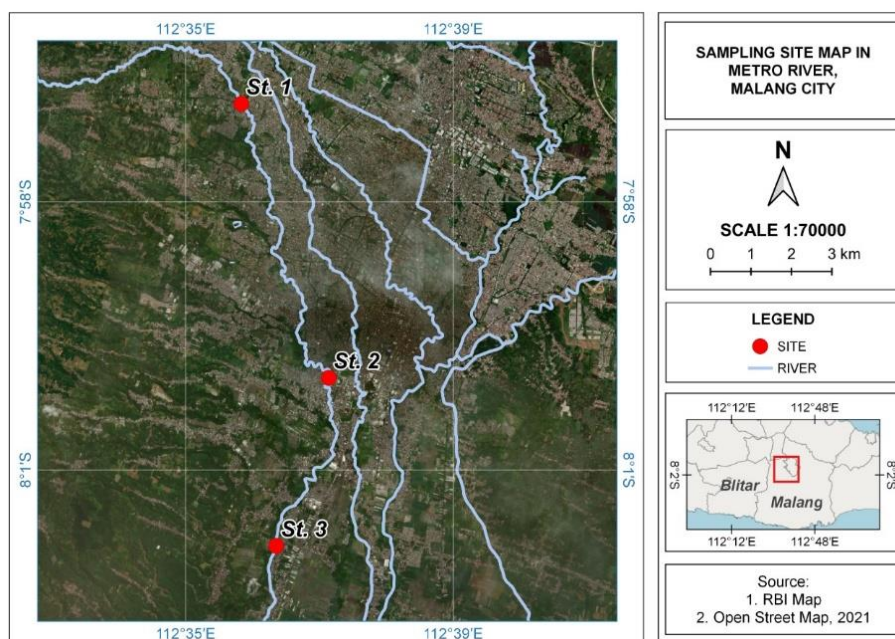


Figure 1. Sampling location

River water (16 L) was taken and filtered using a plankton net to 250 mL of sampling bottle. The filtered water was placed in a plastic bottle and then brought to the laboratory in a coolbox with a temperature of approximately 4°C. The biofilm used in this study is a biofilm that grows naturally on rocks in the Metro River. The biofilm was removed from the rock surface by gently brushing the biofilm into 50 mL distilled water. The biofilm suspension obtained was collected in a plastic container and put into a coolbox (4°C) and then brought to the laboratory.

Microplastic Analysis

The biofilm suspension or river water was filtered using a 5 mM and 0.1 mm stainless steel mesh sieve to obtain microplastics. The resulting particles were then dried using an oven (90°C) for 24 hours. A total of 20 mL of a solution of 0.05 M Fe and 30% H₂O₂ were added to a glass beaker containing the dried particles. Next, 6 g reagent grade NaCl was added to every 20 mL of suspension obtained. Then, the suspension was put into a density separator. The resulting precipitate was discarded, while the supernatant was used to analyze the microplastic content. The supernatant was filtered using Whatman filter paper, and then the presence of microplastics on the filter paper was observed using a microscope (Olympus BX50).

RESULT AND DISCUSSION

Water parameters

Measurement of water quality parameters was carried out in this study included temperature, pH, dissolved oxygen, and flow velocity. These physicochemical parameters affect the distribution and abundance of microplastics in the waters. The results of measuring water quality parameters can be seen in Table 1.

This study's water quality measurement suggested that the pH value at each station ranged from 7.5-7.6. The pH value of waters is influenced by temperature, dissolved oxygen, the biological activity of aquatic organisms, and ions. The biological activity of aquatic organisms such as respiration will increase CO₂ in the waters. The pH will decrease due to increased H⁺ ions released into the waters [11].

The dissolved oxygen in this study ranged from 6.5-7.7 ppm. Aquatic organisms need dissolved oxygen for metabolic processes. Aerobic oxidation of organic and inorganic matter in waters also requires the presence of dissolved oxygen. The primary source of dissolved oxygen is diffusion from the air and photosynthetic products of aquatic organisms [12].

The temperature at each station ranged from 25-26°C. Factors that cause temperature differences are currents and turbulence in the river's upstream, middle, and downstream areas. The downstream area gets a high intensity of sunlight. The heat exchange process between water and air downstream is more remarkable, increasing temperature [13]. Water temperature will affect the process of plastic degradation into microplastics (thermal degradation). The high-temperature value will accelerate the degradation process so that the abundance of microplastics in the waters is high [14]. However, plastic degradation is not only triggered by plastic damage as a result of high temperatures but also due to thermodynamic effects such as being in running water at a temperature of 15°C to 28°C [15].

The current velocity at each station is different and ranges from 0.25-0.5 m.s⁻¹. Water quality in the form of current velocity affects spreading organisms, minerals, and dissolved gases in the waters [14]. Several factors affect river flow speed, namely water discharge, materials, such as stones, mud, and sand found in the river body [16]. The relationship between current velocity and the abundance of microplastics in the waters is unidirectional. Hence, if the current velocity is high, the abundance value of microplastics also increases [17].

Microplastics Abundance

The identification of microplastics that was carried out in this study found several types of microplastics, namely films, fibers, fragments, and beads. These types of microplastics come from different sources and amounts. The four types of microplastics in this study can be seen in Figure 2.

Table 1. The results of water quality parameter measurement

Station	pH	DO (mg.L ⁻¹)	Temperature (°C)	Water Flow velocity (m.s ⁻¹)
Station 1	7.5 ± 0.007	7.7 ± 1.2	25 ± 1.4	0.5 ± 0.1
Station 2	7.5 ± 0.007	6.5 ± 0.4	25 ± 1.4	0.5 ± 0.1
Station 3	7.6 ± 0.7	6.7 ± 0.5	26 ± 0.7	0.25 ± 0.1

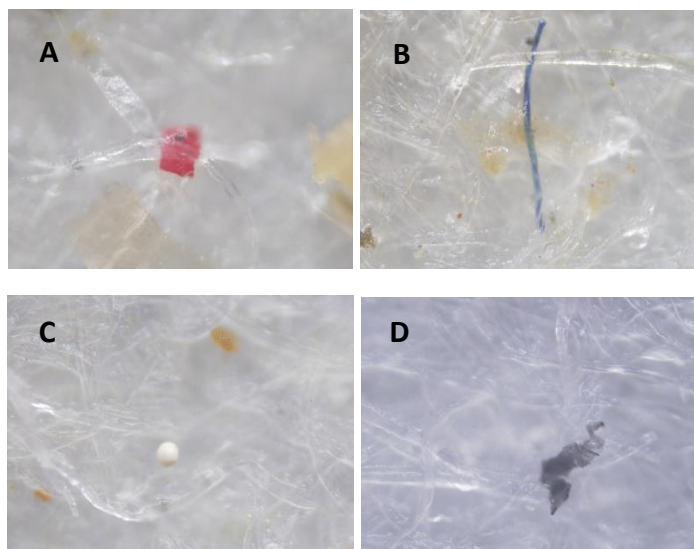


Figure 1. Types of microplastics found in this study (A: Fragments, B: Fiber, C: Beads, D: Film)

Microplastic films have thin and flexible characteristics [18]. The film was formed due to the degradation or pieces of plastic bags [19]. Microplastic fragments are the most commonly found in waters. The highest abundance after fragments is the type of film. The abundance is due to the low density of the film so it was easy to be distributed carried by the current [20].

Fiber has a physical shape resembling a thread and consists of various colors such as black, red, and blue. Fragments have an irregular rectangular physical shape that originates from a larger piece of plastic. Fragments are found in several colors, namely blue, red, green, and brown. Black and white film type microplastics in the form of a plastic sheet [21]. Microplastic beads come from the cosmetic industry and body care products such as soap, shampoo, toothpaste, face masks [22].

Sources of microplastic pollution at station 1 are from household activity waste and runoff from agricultural activities. Microplastics fiber types come from clothing, cloth, raffia, plastic sacks, and others waste disposal from settlements and agriculture [23]. The Station 2 river flow area contained plastic food packaging waste, crackle bags, baby diapers. The waste was intentionally or unintentionally entered the waters. Film-type microplastics have the physical form of thin sheets sourced from plastic bags or other materials made of single-use plastic [24]. Waste disposal at station 3 comes from household and various industrial activities, namely cigarette factories and sugar factories. The packaging or materials used during the

production process at the factory can be a source of fragments of microplastics contaminants. Microplastic fragments come from jars, buckets, plastic bottles, pieces of irrigation pipes, and others from human activities [25].

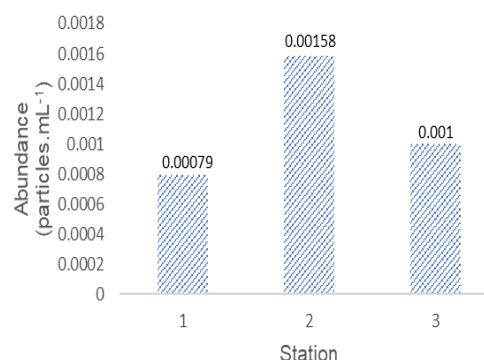


Figure 2. The abundance of microplastics in water

The abundance of microplastics in the water samples obtained in this study differed at each sampling station (Fig. 3). The abundance values of microplastics at each station were as follows; Station 1 was 0.00079 particles.mL⁻¹, Station 2 was 0.00158 particles.mL⁻¹, Station 3 was 0.001 particles.mL⁻¹. The highest abundance of microplastics was at Station 2 because the location was influenced by waste from anthropogenic activities from densely populated settlements. The increasing human activity is directly proportional to the amount of waste produced. The existence of residential areas in the area around the river allows the waste from household activities to be directly discharged into the waters [26].

The abundance of microplastics inside the biofilm materices obtained in this study was different at each sampling station, as shown in Figure 4. The abundance values of microplastics at each station were as follows. Station 1 has as many as 9.5 particles.g⁻¹, Station 2 as many as 9.8 particles.g⁻¹, Station 3 as much as 9.5 particles.g⁻¹. Biofilm is defined as a community of autotrophs and heterotrophs covered by an organic matrix and live on substrates in water. The biofilm-forming microbial community consists of bacteria, algae, fungi [27].

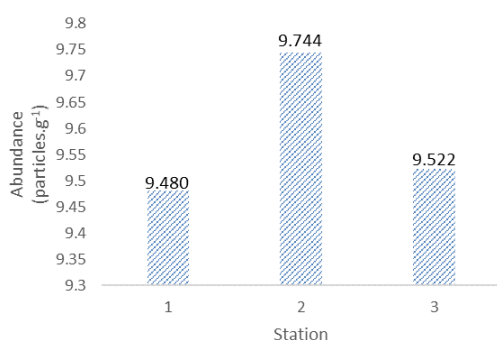


Figure 3. The abundance of microplastics in biofilm matrices

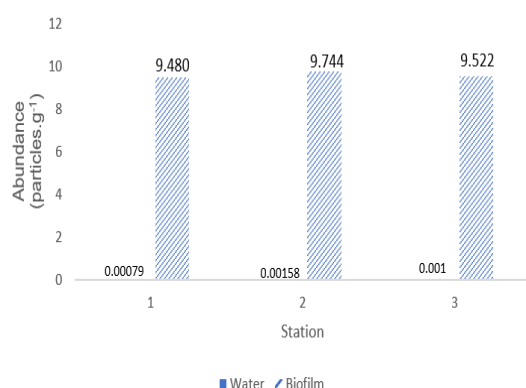


Figure 4. Comparison of microplastic abundance in water and biofilm (comparisons are made in grams assuming 1 mL of water is equivalent to 1 g of water)

In order to compare the abundance of microplastics in the biofilm matrix and water, the abundances of microplastics in the biofilm and river water around the biofilm were compared (Fig. 5). This comparison assumes that 1 mL of water is equivalent to 1 g of water. The results showed that the abundance of microplastics in the biofilm was ten times higher than the abundance of microplastics in water at each sampling station. This higher number may be because biofilms can adsorb microplastics in

adsorption. Because of this ability, biofilms can be used as biosorbents for various pollutants, including microplastics [28]. Biosorption can occur by involving physicochemical processes between pollutants and biofilm polymers through ion exchange mechanisms and electrostatic interactions [29].

This study shows that the biofilm matrix can withstand microplastics. This ability is also found in other biomass in aquatic ecosystems such as fish, aquatic invertebrates, and algae [30]. The results of this study strengthen knowledge to develop biofilms in eco-aquatic technology, especially in assessing the presence of pollutants in aquatic ecosystems.

Microplastic as an emerging pollutant should be monitored for its existence [31]. The monitoring requires agents that are relatively easy to find in all aquatic ecosystems, which is biofilm is an alternative [28,32]. Further studies related to the presence and interaction of microplastics with aquatic organisms are needed to a better understanding of the presence and impact of microplastics in aquatic ecosystems, especially on biofilms as the predominant habitat of aquatic microbes [33]. The interaction of aquatic microbes and microplastics as emerging pollutants will increase opportunities to use microbes to manage and conserve aquatic ecosystems.

CONCLUSION

This study was conducted to identify the abundance of microplastics in the Metro River, Malang. This study showed that river water and biofilm in metro rivers contain microplastics. The types of microplastics at all sampling stations consisted of fiber, fragments, films, and beads. The abundance of microplastics in river water ranged from 0.8 - 1.61 particles.mL⁻¹ and inside the biofilm matrix ranged from 7.4 - 9.5 particles.g⁻¹. Assuming 1 mL of water is equivalent to 1 gram of water, the microplastic content in biofilm is ten times higher than in river water. This study showed that microplastics, widely seen as emerging pollutants, must receive attention in managing river ecosystems in Indonesia.

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Synergism of *Lecanicillium lecanii* (Zimm) and *Chromolaena odorata* L. Leaf Extract to Control *Aphis gossypii* (Glover) in Chili Plants

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Abstract

The purpose of the study was to determine the effect of the appropriate and effective application of the *L. lecanii* fungus and *C. odorata* L. leaf extract in increasing the mortality of *A. gossypii*. The study was divided into two stages. The first stage was *in vitro* test of *L. lecanii* synergism plus *C. odorata* L. leaf extract in PDA media with four treatments, namely LK₀= *L. lecanii* 10⁷ conidia.mL⁻¹ plus 0% *C. odorata* L. leaf extract, LK₁= *L. lecanii* 10⁷ conidia.mL⁻¹ plus 10% *C. odorata* L. leaf extract, LK₂= *L. lecanii* 10⁷ conidia.mL⁻¹ plus 25% *C. odorata* L. leaf extract; LK₃= *L. lecanii* 10⁷ conidia.mL⁻¹ plus 40% *C. odorata* L. leaf extract. The second stage was the toxicity test of the application of *L. lecanii* suspension and *C. odorata* L. leaf extract on mortality of *A. gossypii*. The toxicity test was based on the results of the synergism test, where the addition of *C. odorata* L. leaf extract to *L. lecanii* growing media showed incompatible results. Therefore, the toxicity test was carried out separately with five treatments, namely H₀= Control (aqua dest); H₁= Conidia suspension *L. lecanii* 10⁷ conidia mL⁻¹; H₂= 10% *C. odorata* leaf extract; H₃= 25% leaf extract of *C. odorata*; H₄= 40% *C. odorata* leaf extract. The results showed that the compatibility test of *L. lecanii* with leaf extract of *C. odorata* L. was incompatible and classified as toxic. The addition of *C. odorata* L. leaf extract in concentrations of 10%, 25%, and 40% could significantly inhibit colony growth, sporulation, and conidia viability of *L. lecanii*, with a higher level of inhibition as the concentration of *C. odorata* L. leaf extract, was added. The toxicity test of a separate application of *C. odorata* L. leaf extract and *L. lecanii* suspension had a significant effect on mortality of 3rd instar nymph *A. gossypii*, with the highest mean mortality found in a single application of 40% *C. odorata* L. leaf extract with an average mortality of 100% at 96 HAA(Hours After Application) observations.

Keywords: *A. gossypii*, *C. odorata* L. leaf extract, *L. lecanii*, synergism.

INTRODUCTION

Chili is one of the important horticultural commodities in Indonesia. Data on the projected level of chili consumption in Indonesia is expected to continue to increase, seen from 2016 at 2.90 kg.capita⁻¹, an increase of 3.05 kg.capita⁻¹ in 2019 [1]. The increasing chili consumption has not been matched by a stable production level and low productivity. One of the factors causing the low productivity of chili is the presence of pests and diseases [2]. One of the pests that often cause damages to chili plantations is aphid from the *Aphis gossypii* species.

Aphis gossypii is a commonly found pest on chili plants because this pest has a wide host range, such as the Fabaceae, Solanaceae, Cucurbitaceae, and Asteraceae families. This pest has a wide distribution area from tropical to temperate climates, so *A. gossypii* can survive on almost all cultivation plants and populations of aphids that always exist in every season [3]. Aphids can cause damage to plants because they attack by sucking plant fluids, causing loss of plant nutrients and damaged plant cells and

tissues [4]. Aphids also produce honeydew in the form of a sweet liquid that can cover the surface of plant leaves, resulting in inhibition of the photosynthesis process. *Aphis gossypii* also acts as a vector for disease-causing viruses, such as CMV and ChiVMV viruses in chili plants, with symptoms of stunted plants and fall before fertilization [5].

The losses caused by *A. gossypii* attacks are in line with the level of population on the host plant. The higher the *A. gossypii* population, the higher the damage to plants. A high and uncontrolled aphid population can cause damage to chili plants up to 65% [6]. Control of aphids currently generally still uses synthetic insecticides, which have the potential to cause the death of natural enemies, pest resistance, and environmental pollution [7]. In addition, the use of chemical pesticides also has affected human health because of the chemical content of pesticides left on agricultural products [8]. There is a need for alternative pest control that is more environmentally friendly and safe for consumer health, one of which is by using biological agents or botanical pesticides.

The fungus *Lecanicillium lecanii* is an entomopathogenic fungus that has the potential to control insects. The fungus *L. lecanii* can infect several types of host insects from the orders

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Orthoptera, Hemiptera, Lepidoptera, Thysanoptera, and Coleoptera [9]. The use of the fungus *L. lecanii* with a conidia density of 5×10^7 mL⁻¹ is pathogenic and causes *A. gossypii* mortality with an average mortality of $59.00 \pm 3.83\%$ [10].

Chromolaena odorata L. plant has potential as a botanical pesticide, whereas *C. odorata* L. contains active sesquiterpene compounds effective against termite mortality [11]. Weed of *C. odorata* L. is also reported to have pyrrolizidine alkaloids as active compounds which are toxic [12]. The application of *C.odorata* L. leaf extract with a concentration of 40% was effective in killing *A. gossypii* with 100% mortality, and the application of botanical pesticides of *C. odorata* L. leaf extract could cause *A. gossypii* to move slowly and eventually die [13]. *Chromolaena odorata* L. leaf extract with a concentration of 80 g.L⁻¹ of water was effective in controlling *Aphis gossypii* (Glover) with total mortality of 95% at an LC₉₅ of 7.7% [14].

The application of pest control combination using biological agents and botanical pesticides has been done before. The combination of vegetable insecticides such as *Aglaia odorata*, *Annona squamosa*, and *Jatropha curcas* is synergistic because it can increase the growth of *L. lecanii* colonies. It also increased the control efficacy of brown ladybug eggs up to 77% compared to the single application [15]. The combination of the fungus *M. anisopliae* with 2 mL and 3 mL of *babadotan* leaf extract can increase the mortality of *N. viridula* compared to a single application [16]. The combination of entomopathogenic fungi and botanical pesticides was able to increase pest mortality. Therefore, the purpose of this study was to determine the effect of the most effective application of the entomopathogenic fungus *L. lecanii* and *C. odorata* L. leaf extract in increasing the mortality of aphids (*A. gossypii*) on chili plants.

MATERIAL AND METHOD

The research was carried out at the HPT Laboratory, Faculty of Agriculture, University of Jember, and in the Patrang greenhouse, Jember Regency. The study started from February to September 2021.

The study consisted of two stages arranged in a completely randomized design. The first stage was the compatibility test of the fungus *L. lecanii* and *C. odorata* L. leaf extract. It was carried out *in vitro* with the technique of mixing *C. odorata* L. leaf extract concentrations of 10%, 25%, and 40%

on PDA media through colony growth calculations, sporulation, and viability with four treatments and five replications. The second stage was the toxicity test on the application of the fungus *L. lecanii* and *C. odorata* L. leaf extract to the mortality of *A. gossypii* with five treatments and four replications. The data obtained were analyzed by ANOVA and continued with the DMRT test with a 95% confidence level.

Data Collection

Compatibility Test

Colony Diameter Growth *L. lecanii*

Measuring the diameter of the fungus colonies on the 7th day after application (DAA) with a ruler. The calculation was done by making vertical and horizontal lines on the petri dish then the measurement results were entered into the following Equation 1 [17]:

$$D = \frac{d_1 + d_2}{2} \dots\dots\dots 1$$

Description:

D = diameter of *L. lecanii* colony (cm)
d₁ = vertical diameter of *L. lecanii*
d₂ = horizontal diameter of *L. lecanii*

The percentage decrease in colony growth was calculated by the Equation 2 [18]:

$$Nr = \frac{N_1 - N_2}{N_1} \times 100\% \dots\dots\dots 2$$

Description:

Nr = percentage decrease in colony growth
N₁ = growth of fungal colonies on media that was not given *C. odorata* L. leaf extract
N₂ = growth of fungal colonies on media that was given *C. odorata* L. leaf extract

Sporulation or Number of Conidia

Count number of spores in each treatment after the fungus was conducted at the 7th DAA. Calculation of the conidia number was carried out by harvesting conidia in a petri dish first, harvesting conidia by adding 5 mL of aqua dest then leveling and placing it in a test tube containing 5 mL of aqua dest, then vortexing the suspension and taking 1 mL of *L. lecanii* suspension and placing it on a hemocytometer.

$$J = \frac{t \times d}{0.25 \times n} \times 10^6 \dots\dots\dots 3$$

Description:

J = calculated conidia density (conidia mL⁻¹)
t = the number of conidia in the calculated sample box
d = dilution rate
n = number of observed sample boxes (80)
0.25 = correction factor

The number of conidia was counted using a compound microscope with a magnification of 400 times. The conidia were counted in five sample boxes. The results of observations of the number of conidia were added up and calculated by the Equation 3 [19]. Meanwhile, the percentage decrease in sporulation was calculated by the Equation 4 [18]:

$$Sr = \frac{S_1 - S_2}{S_1} \times 100\% \dots\dots\dots 4$$

Description:

- Sr = percentage decrease in sporulation
- S₁ = the number of spores produced by the fungus on media not given *C. odorata* L. leaf extract (control)
- S₂ = the number of spores produced by the fungus in the media given the *C. odorata* L. leaf extract

Viability or Number of Germinating Conidia

Harvesting conidia on PDA media according to the treatment, then taking a suspension of *L.lecanii* conidia and incubating on glass slides and placing them on a damp tissue placed on a tray and covered with plastic wrap for 24 hours. Conidia germination was observed using a microscope with a magnification of 400 times. Germination percentage was calculated from 100 conidia, where conidia were said to germinate if the length of the germination tube had exceeded the diameter of the conidia. Calculation of germination (viability) of spores is calculated using the Equation 5 [20]:

$$V = \frac{g}{g+u} \times 100\% \dots\dots\dots 5$$

Description:

- V = conidia germination
- g = number of germinated conidia
- u = number of conidia that did not germinate

The percentage decrease in sporulation was calculated by the Equation 6 [18]:

$$Mr = \frac{M_1 - M_2}{M_1} \times 100\% \dots\dots\dots 6$$

Description:

- Mr = percentage decrease in germination
- M₁ = germination of conidia in media that was not given *C. odorata* L. leaf extract (control)
- M₂ = germination of conidia in media that was given *C. odorata* L. leaf extract

Compatibility Value Calculation

The results of the compatibility observations are entered into the T formula as following Equation 7 [18]. The T value is divided into categories, namely 0-30 very toxic, 31-45 toxic, 46-60 less toxic, and >60 non-toxic (compatible).

$$T = \frac{\{20(PK)+80(SP)\}}{100} \dots\dots\dots 7$$

Description:

- T = compatibility value (%)
- PK = relative value of treatment colony growth compared to control (%)
- SP = relative value of treatment sporulation compared to control (%)

Toxicity Testing of *L. lecanii* and *C. odorata* L. Leaf Extract against *A. gossypii*

Observations were made every 24 hours for six days after application. Each experimental unit used 3rd instar aphid nymph insects. As many as 20 individuals were placed under the surface of chili leaves aged 1 week after planting (WAP) using a soft brush. Aphid inoculation is done using a soft brush on the underside of the chili plant leaves. Applications were carried out by spraying with a hand sprayer, *L. lecanii* conidia density 10⁷ conidia.mL⁻¹ aqua dest, and *C. odorata* L. leaf extract at 10% concentration; 25% and 40%, as well as for the control treatment using aquadest. Before application, calibration was carried out to determine the volume of spray used, which was 10 mL for each treatment. The spray volume used was 10 mL for each treatment of *L. lecanii* conidia suspension and *C. odorata* leaf extract, and also aquadest for the control treatment. The percentage of insect mortality was calculated by the Equation 8 [21]:

$$M = \frac{n}{N} \times 100\% \dots\dots\dots 8$$

Description:

- M = mortality (%)
- n = number of dead *A. gossypii*
- N = number of tested *A. gossypii*

RESULT AND DISCUSSION

Compatibility of *L. lecanii* and *C.odorata* L. Leaf Extract

The results showed that the addition of *C. odorata* L. leaf extract on the growth medium of the fungus *L. lecanii* had a very significant effect on the growth of colony diameter, sporulation, and the viability of the fungus *L. lecanii* (P<0.05). Table 1 shows that the addition of *C. odorata* L. leaf extract significantly inhibited the growth of the diameter of the fungus *L. lecanii*.

Colony Diameter of *L. lecanii*

Colony growth of *L. lecanii* without *C. odorata* L. leaf extract addition had the highest average diameter of 5.27 cm, compared to the diameter of *L. lecanii* colonies with *C. odorata* L. leaf extract. The addition of *C. odorata* L. leaf extract at a concentration of 10% showed an average

diameter of *L. lecanii* of 2.48 cm, and the percentage decrease in diameter was 52.94%. Then the addition of *C. odorata* L. leaf extract with a concentration of 25% showed an average value of *L. lecanii* diameter of 2.34 cm with a percentage a decrease in the diameter of 55.60%, while the addition of *C. odorata* L. leaf extract with the highest concentration was 40% showed that the inhibition of the growth of *L. lecanii* colonies was much greater, for 2.23 cm, which means that the growth of *L. lecanii* colonies decreased by 57.69% (Fig.1).

The inhibition of the growth of *L. lecanii* colonies was thought to be due to the content of *C. odorata* L. leaves, which act as fungistatic antimicrobial compounds that can temporarily inhibit the growth of the fungus *L. lecanii*. Leaf extract of *C. odorata* L. has potential as an antifungal because it has antifungal activity against the growth of *Aspergillus flavus* fungus [22]. The addition of *C. odorata* L. leaf extract with a concentration of 0.5% was effective in suppressing the growth of the *Colletotrichum capsici* and had a very significant effect on the colony diameter and the percentage of inhibition of *C. capsici* [23]. Therefore, it is better not to

combine it with *C. odorata* L. leaf extract because the *C. odorata* L. leaf extract has antifungal compounds that can inhibit the colony growth of the *L. lecanii* fungus.

The effectiveness of an antifungal substance is influenced by the concentration of the substance given [24]. It is in line with the observations which showed that increasing the concentration of *C. odorata* L. leaf extract results in a high content of active ingredients. It has function as antifungals so that the ability to inhibit fungal growth will be greater. So, the higher the concentration of *C. odorata* L. leaf extract given will also increase the anti-fungal properties that can inhibit the growth of the fungus *L. lecanii*.

Sporulation, and Viability of *L. lecanii*

Table 1 shows that the addition of *C. odorata* L. leaf extract in various concentrations also significantly affected the number of spores (sporulation) of *L. lecanii* conidia. The addition of *C. odorata* L. leaf extract significantly reduced the ability of the fungus to sporulate compared to the control treatment without the addition of *C. odorata* L. leaf extract.

Table 1. Mean Diameter, Sporulation, and Viability of *L. lecanii* Fungus Colonies with the Addition of *C. odorata* L. Leaf Extract

Treatment	Diameter 7 th DAA (cm)±SD	Decrease in Diameter (%)	Sporulation of conidia (10 ⁸)	Decrease in Sporulation (%)	Conidia viability (%)±SD	Decrease in viability (%)
LK ₀	5.27 ± 0.92 ^a	0	3.66 ± 1.01 ^a	0	75.20 ± 2.86 ^a	0
LK ₁	2.48 ± 0.21 ^b	52.94	1.52 ± 0.24 ^b	58.47	69.00 ± 1.58 ^b	8.24
LK ₂	2.34 ± 0.16 ^b	55.60	1.32 ± 0.19 ^b	63.93	67.60 ± 0.89 ^b	10.11
LK ₃	2.23 ± 0.29 ^b	57.69	1.27 ± 0.26 ^b	65.30	67.00 ± 1.87 ^b	10.90
sig	0.000		0.000		0.000	

Note: LK₀ = *L. lecanii* + *C. odorata* L. Extract 0%, LK₁ = *L. lecanii* + *C. odorata* L. Extract 10%, LK₂ = *L. lecanii* + *C. odorata* L. Extract 25%, LK₃ = *L. lecanii* + *C. odorata* L. Extract 40%; SD = Standard Deviation.

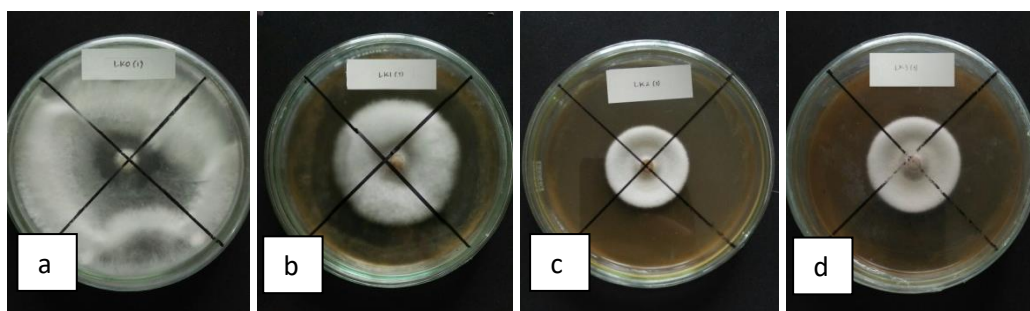


Figure 1. Colony growth of *L. lecanii* at 7 days after inoculation (DAI) with the addition of *C. odorata* L. leaf extract (a) at 0% concentration, (b) 10% concentration, (c) 25% concentration, (d) 40% concentration.

In LK₁ treatment, the addition of *C. odorata* L. leaf extract with a concentration of 10% significantly inhibited *L. lecanii* from sporulating with a decrease of 58.47%. In LK₂ treatment, the addition of *C. odorata* L. leaf extract with a concentration of 25% has a sporulation value of

1.52×10^8 conidia.mL⁻¹, with a decreased percentage of sporulation reaching 63.93%. And in LK₃, the addition of *C. odorata* L. leaf extract with a concentration of 40% gave a much lower effect, with a sporulat of only 1.27×10^8 conidia.mL⁻¹ with a decrease in sporulation

percentage of 65.30%. The higher the concentration of *C. odorata* L. leaf extracts given to the growth medium, the lower the number of *L. lecanii* conidia produced.

Leaf extract of *C. odorata* L. has antifungal properties and is effectively used as a fungicide in suppressing the growth and formation of *Colletotrichum musae* spores [25]. The antifungal properties of *C. odorata* L. leaves are thought to be caused by the content of secondary metabolites in *C. odorata* L. leaves. The results of phytochemical analysis of *C. odorata* L. leaves contain secondary metabolites such as flavonoids, saponins, terpenoid, steroids, phenolics, and tannins [26]. Plants containing secondary metabolites such as alkaloids, flavonoids, tannins, saponins, and glycosides have potential as antifungals, with different antifungal activity mechanisms between one compound and another [27].

Saponins are secondary metabolites that act as antifungals and antimicrobials [28]. Saponins can inhibit the growth of the fungus *L. lecanii*, where saponins as antifungals work by disrupting the stability of cell membranes resulting in the lysis of microbial cells. Saponins will reduce the surface tension of the fungus so that it can increase cell permeability which causes cell leakage, and intracellular compounds in cells will come out [29]. Saponin compounds in the Ambon banana stem also have an antifungal activity that inhibits the growth of the fungus *Candida albicans* [30].

The mechanism of action of flavonoids in inhibiting fungal growth is by causing interference with the permeability of fungal cell membranes. The hydroxyl group contained in flavonoid compounds causes changes in organic components and nutrient transport, which will eventually lead to toxic effects on fungi [31].

The content of tannin metabolites also acts as an antifungal. The antifungal mechanism caused by tannin compounds inhibited chitin synthesis, which is functional in the fungal cell walls formation and can damage cell membranes so that fungal growth is inhibited. Tannin compounds are lipophilic, which means they are easily attached to fungal cell walls [32]. Terpenoid compounds can also inhibit the growth of fungi through the mechanism of decreasing the permeability of the cell membrane of microorganisms; where the compounds contained in terpenoids are bound to protein and lipid molecules so that they can

affect the physiological function of cell membrane proteins and enzyme proteins [33].

Viability of *L. lecanii*

The addition of *C. odorata* L. leaf extract with various concentrations was also significantly affected the conidia germination (viability) of the fungus *L. lecanii*. The addition of *C. odorata* L. leaf extract significantly reduced the ability of the fungus to germinate compared to the control treatment without the addition of *C. odorata* L. leaf extract. In LK₁ treatment, the addition of *C. odorata* L. leaf extract with a concentration of 10% significantly inhibited *L. lecanii* germination with a decrease of 8.24% with a viability value of 69.00%. It was followed by LK₂ treatment with the addition of *C. odorata* L. leaf extract with a concentration of 25%, which has a viability value of 67.60% with a decreasing percentage of germination 10.11%. Last, the LK₃ treatment (*C. odorata* L. leaf extract with a concentration of 40%) was showed a viability value of 67.00% and a percentage decrease in the germination of 10.90%. The higher the concentration of *C. odorata* L. leaf extract added, the lower the germination rate of conidia *L. lecanii*.

Conidia germination rate and high sporulation can be seen from good fungal physiology, where high conidia germination rate will also increase the virulence ability of the fungus [34]. Viability is positively correlated with the level of fungal infection, where the higher the viability of the spores, the faster the fungus produces spores, thus causing the process of infection in insects to be faster [35]. The faster the occurrence of insect infection by fungi, the faster the process of insect death will be [36].

Based on the calculation of the T value, the classification of the compatibility of *C. odorata* L. leaf extract with the fungus *L. lecanii* combination treatment showed a classification at a toxic level. It meant that it was not compatible with the fungus *L. lecanii* (Table 2). The results showed that the treatment with the addition of *C. odorata* L. leaf extract in growth media with concentrations of 10%, 25%, and 40% showed compatibility values at the toxic level against the fungus *L. lecanii*. It means that the addition of *C. odorata* L. leaf extract was not compatible with the fungus *L. lecanii*. This compatibility value indicated that the leaf extract of *C. odorata* L. and the fungus *L. lecanii* was not compatible, supported by data on decreased colony growth, sporulation, and significantly decreased germination of conidia of the fungus *L. lecanii*.

Table 2. Leaf Extract of *C.odorata* L. Compatibility Classification with Entomopathogenic Fungi *L. lecanii*

Treatment of <i>L. lecanii</i> (conidia.mL ⁻¹ aqua dest) with EC%	%T (Compatibility Value)	Compatibility Level Classification
LK ₁ (<i>L. lecanii</i> + <i>C. odorata</i> L. Leaf Extract 10%)	42.64	Toxic (Not Compatible)
LK ₂ (<i>L. lecanii</i> + <i>C. odorata</i> L. Leaf Extract 25%)	37.73	Toxic (Not Compatible)
LK ₃ (<i>L. lecanii</i> + <i>C. odorata</i> L. Leaf Extract 40%)	36.22	Toxic (Not Compatible)

Note: T is the value of the compatibility test results

Toxicity of Fungus *L. lecanii* and *C. odorata* L. Leaf Extract against Mortality of Instar-3 Nymph *A. gossypii*.

From the compatibility test, it was found that the treatment with the addition of *C. odorata* L. leaf extract showed incompatible results, so the toxicity test was carried out separately. The results of the toxicity test showed the separate application of the suspension of the *L. lecanii* fungus with a density of 10⁷ conidia.mL⁻¹ aqua dest and the addition of various concentrations of *C. odorata* L. leaf extract gave a significantly different effect on the mortality of 3rd instar nymph *A. gossypii* (Table 3), with a significance value of p<0.05. The mortality percentage of *A. gossypii* nymphs will increase along with the increasing concentration of *C. odorata* L. leaf extract given, where the higher the concentration level of *C. odorata* L. leaf extract, the more effective it is in killing 3rd instar nymphs of *A. gossypii*, with the best concentration in the H₄ treatment using *C. odorata* L. leaf extract at a concentration of 40%, with an average mortality of 100% at 96 HAA. In the control treatment that was sprayed with aquadest, none of the aphids nymphs died.

Characteristics of *A. gossypii* that died due to the application of *L. lecanii* showed different symptoms from the death due to the application of *C. odorata* L. leaf extract. It was based on the observation valuation of aphids death symptoms caused by the application of *L. lecanii* suspension. The valuation was seen from the change in the color of the aphid body, which was black and

stiff, the aphid body hardened and hyphae appeared on the body surface, and within a few days, the body surface of the aphids would be covered with mycelium (Fig. 2). Insect death within a few days after application of *L. lecanii* suspension showed insect's body becomes hard due to the fungus attack on all tissues and fluids in the insect's body until it runs out, which makes the insect turn black and stiff. Then the insect's body will slowly be covered with mycelium [37]. Observation of the symptoms of aphids' death caused by the fungus *L. lecanii* was also carried out using the Koch Postulate test to ensure that the pathogen that caused the death of aphids came from the fungus *L. lecanii*.



Figure 2. Nymphal microscopic observation results of *A. gossypii* instar-3 infected with the *L. lecanii* grew hyphae on the body surface of aphids.

The fungus *L. lecanii* will kill host insects by digesting the host's body tissues as a source of food or nutrition and producing toxic substances that kill insect pests. The toxic compounds were in the form of *dipecolinic acid* and *cyclosporin* which have insecticidal properties so that they have a very toxic effect when applied to the host insect [10].

Table 3. Average mortality of *A. gossypii* after application of entomopathogenic fungus *L. lecanii* and botanical pesticide *C. odorata* L. leaf extract

Treatment	On observation...HAA (%) (\bar{x})					
	24	48	72	96	120	144
H ₀	0.00 ^d	0.00 ^d	0.00 ^e	0.00 ^e	0.00 ^d	0.00 ^d
H ₁	3.75 ^d	10.00 ^d	22.50 ^d	41.25 ^d	53.75 ^c	62.50 ^c
H ₂	38.75 ^c	53.75 ^c	68.75 ^c	75.00 ^c	80.00 ^b	83.75 ^b
H ₃	60.00 ^b	68.75 ^b	85.00 ^b	87.50 ^b	95.00 ^a	96.25 ^a
H ₄	85.00 ^a	95.00 ^a	98.75 ^a	100.00 ^a	100.00 ^a	100.00 ^a
Sig.	0.000	0.000	0.000	0.000	0.000	0.000

Description: H₀ = Control (aquadest), H₁ = *L. lecanii* suspension 10⁷ conidia.mL⁻¹, H₂ = 10% concentration of *C. odorata* L. Leaf Extract, H₃ = 25% concentration of *C. odorata* L. Leaf Extract, H₄ = 40% concentration of *C. odorata* L. Leaf Extract; HAA= Hours After Application

The fungus *L. lecanii* infects the host through the formation of germ tubes by conidia on the cuticle surface, which will be used to penetrate the insect cuticle, then the fungus will enter through the integument and damage the physiology of the insect host. The surface of the host insect's body is hydrolyzed more quickly by enzymes produced by fungi, causing insect death [39]. Observations on the characteristics of 3rd instar nymphs of *A. gossypii* that died due to the application of *C. odorata* L. leaf extract showed that at first, the insects experienced changes in behavior and tended to be less active or passive when touched with a brush. The insect's body also turned dark or blackish brown (Fig.3). Application of botanical pesticides from *C. odorata* L. leaves can cause the movement of *A. gossypii* pests to be slow and the lice will die over time [13].



Figure 3. Nymph Death Symptoms *A. gossypii* instar 3 after *C. odorata* L. Leaf Extract Application; (a) microscopic; (b) macroscopic.

Leaf extract of *C. odorata* L. can cause death in *A. gossypii* because of its active ingredient compounds, *Pyrrolizidine alkaloids* which are toxic [12]. The content of *Pyrrolizidine alkaloids* in *C. odorata* L. leaves can cause the plant to have a pungent smell and taste bitter, so it is insect repellent [26]. In addition, the content of secondary metabolites contained in *C. odorata* L. leaves can also cause death in aphids, with a characteristic the insect's body turned dark or blackish brown, and activity change that becomes passive. Leaves of *C. odorata* L. contain several secondary metabolites such as saponins, tannins, flavonoids, alkaloids, and phenolics [27]. The content of alkaloid and flavonoid compounds in *C. odorata* L. leaves can be toxic to insects and inhibit appetite or *antifeedant* for pests. The compounds were acted as stomach poisons so that aphids poisoned over time and eventually died.

CONCLUSION

The compatibility test of the fungus *L. lecanii* with *C. odorata* L. leaf extract showed incompatibility and was classified as toxic. The

addition of *C. odorata* L. leaf extract at concentrations of 10%, 25%, and 40% could significantly inhibit colony growth, sporula, and viability of *L. lecanii* conidia, with a higher level of inhibition as the concentration of *C. odorata* L. leaf extract, was added. Toxicity test of a separate application of *C. odorata* L. leaf extract and *L. lecanii* suspension had a significant effect on mortality of 3rd instar nymph *A. gossypii*, with the highest mean mortality found in a single application of 40% *C. odorata* L. leaf extract with an average mortality of 100% in 96 HAA observations.

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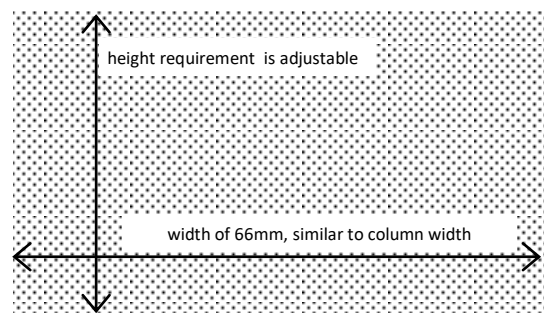


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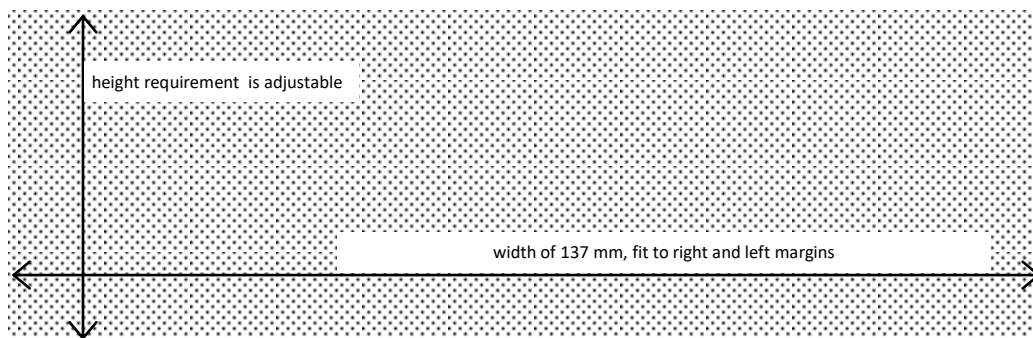


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