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Concentration and Staining Duration Effects of *Clitoria ternatea* Extract on Whole mount Preparate Quality

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Abstract: *Making intact preparations to observe the anatomical structure of plants in biological practicum activities requires effective dyes. Synthetic dyes such as safranin are commonly used, but they are relatively expensive, carcinogenic, and less environmentally friendly. This study aimed to examine the effect of variations in concentration and duration of staining using butterfly pea flower extract (Clitoria ternatea) on the quality of the whole plant preparation Hydrocotyle umbellata. The study was conducted experimentally with a combination of concentration treatment (70%, 80%, and 90%) and duration of staining (15 and 30 minutes). The quality of the preparations was assessed using assessment parameters which included clarity of anatomical structure, colour contrast and colour distribution. The data obtained were analyzed to determine the best treatment combination, as well as compared with safranin-stained and no-staining control preparations. Results showed that increasing concentration and duration of staining had an effect on the quality of the preparation results. The combination of concentrations of 80% of the 15-minute duration of staining gave the best results with the identification of trichomes and cellularcomponents, strong color contrast and even color. Based on these results, butterfly pea flower extract has the potential to be used as an alternative natural dye equivalent to safranin which is safer, more economical, and more environmentally friendly.*

Keywords: *Microtechnique practicum, Clitoria ternatea, Hydrocotyle umbellata, natural dyes, whole mount*

1. INTRODUCTION

Coloring is an important stage in making microscopic preparations because it functions to increase contrast and clarify the anatomical structure of plants so that they can be observed optimally (Kurniawan, 2023). One example of using color in microscopic observations is making whole mount preparations, observing complete plant or animal tissues or organs without cutting (Robbika, 2023). The success of the observations was greatly influenced by the staining technique, which served to increase the visual contrast to allow the anatomical details to be clearly observed under the microscope. So far, synthetic dyes such as safranin have been widely used because of their ability to produce good color contrast, but their use has limitations, including their relatively expensive price and chemical content which is irritative and potentially toxic (Dafrita et al., 2020). This condition makes it difficult for educational institutions that have limited laboratory facilities, so alternative dyes are needed that are safer, more affordable and easier to obtain.

In line with the demands of environment-based science education and the use of local resources, the use of natural dyes is a relevant and important option. One potential ingredient is

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butterfly pea flower (*Clitoria ternatea*), which contains the pigment anthocyanin and is able to bind to plant cell walls. Anthocyanin pigments are flavonoid compounds which, under acidic conditions, are in the form of positively charged and stable flavilium cations (Joseph et al., 2021). Anthocyanins are polar as weak acids that have a low pH (Syifa Unawahi, Asri Widyasanti, 2022). This condition makes it easier for anthocyanins to interact with plant cell walls which are negatively charged bases. Such charge differences allow for charge attraction and hydrogen bond formation, allowing pigments to diffuse into the tissue, adsorb, and persist in the cell wall (Sari, 2020). This mechanism supports color stability and produces clear tissue contrast in botanical preparations.

A number of studies state that butterfly pea flower extract is able to produce colors with adequate levels of contrast in various biological preparations. Nevertheless, the results of previous studies showed non-uniformity of effectiveness based on the variation of the object, tissue thickness, extract concentration and duration of staining. Research by Ningrum et al., (2024) and Aini et al., (2023) reported that low concentrations produced less intense colors, while moderate concentrations showed the most stable results. On the other hand, research Atik et al., (2023) shows that high concentrations can actually inhibit color penetration in bacteria, while Meidevita & Sayekti, (2024) found the opposite result in certain fungal objects. Variations in these findings suggest that the optimal parameters of staining depend on the type of object, staining concentration, anthocyanin pH, and staining duration.

These findings show that there is a scientific gap, namely that there is no research that specifically examines the optimization of the concentration and duration of butterfly pea flower extract coloring in *whole mount* plant preparations, especially in ideal species such as *Hydrocotyle umbellata* which have thin leaves, and are suitable to be observed without cutting (Hamdy et al., 2018). In addition, there have been no studies comparing the results of the combination of treatment with the synthetic dye safranin as a control, so it has not been possible to confirm the feasibility of butterfly pea flower extract as a conventional dye substitution in biological practicums.

Based on this research gap, this study offers novelty in the form of determining optimal coloring parameters through a combination of three levels of extract concentration (70%, 80%, 90%) and two duration of coloring (15 and 30 minutes) in whole mount preparations of *Hydrocotyle umbellata* plants. Evaluation was carried out on visual quality parameters which included clarity of structure, color intensity and uniformity of coloring distribution. This research not only produces scientific information regarding the performance of anthocyanins as plant histology dyes, but also opens up alternative natural dyes that are more cost-effective, environmentally friendly. This study hypothesizes that the combination of butterfly pea flower (*Clitoria ternatea*) extract concentration and staining duration significantly affects the quality of whole mount preparations of *Hydrocotyle umbellata*.

2. RESEARCH METHOD

The type of research used is true experiment with a complete random design (RAL). Using a combination of treatment of butterfly pea flower extract staining concentration and staining duration. Experimental group with a combination of 70%, 80%, and 90% concentrations and 15 minutes and 30 minutes duration of staining. The positive control group with synsthesis dye was safranin and the negative control without staining.

The population and samples used in this study were butterfly pea flower obtained around housing, young *Hydrocotyle umbellata* plants measuring 5-7 cm obtained around campus. The tools used are scalpel, glass beaker, digital scale, flacon bottle, aluminum foil, scalpel, petri dish, object glass, deck glass, watch glass, microscope, camera, rotary evaporator. Materials used are butterfly pea flower, *Hydrocotyle umbellate* plants, 96% ethanol, water, safranin, alcohol, FAA, xylol, aquades, entellan.

The research procedure was carried out in three stages, namely in the first stage of collecting materials and making butterfly pea flower extract, the second stage was making whole mount plant preparations, the third stage was collecting data and analyzing data. The extract preparation stage begins with a drying process for making powder, then soaking using 96% ethanol solvent for 2 x 24 hours. After that, the process of separating the

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anthocyanin compound by evaporating the solvent uses a rotary evaporator. After the extract is obtained, it is continued by making concentration variations of 70%, 80% and 90%.

The whole mount preparation manufacturing stage begins with a fixation process using FAA solution for 1 x 24 hours. Fixation aims to preserve and prevent damage to tissue and cell structures so that they remain as they are. The staining process contained a control group with a 15-minute duration safranin dye, and a concentration treatment group of 70% 15-minute duration, a concentration of 70% 30-minute duration, a concentration of 80% 15-minute duration, a concentration of 80% 30-minute dyration, a concentration of 90% 15-minute duration, and a concentration of 90% 30-minute duration. After staining, there is a clearing process using 50%, 70%, 80%, 90% graded alcohol, and absolute alcohol. Then dehydrate with xylol: graded alcohol. The preparations that have been obtained are observed under a microscope and mounted using entellan to protect the preparations and maintain stability in the long term.

Data collection was carried out by observing the quality of the preparations from microscope photographs using observation sheets. The independent variable in this study consisted of eight treatment groups, namely six combinations of butterfly pea flower extract concentration and staining duration, as well as two control groups (safranin staining and without staining).

The dependent variable was the quality of the whole mount preparations. Operationally, preparation quality was measured based on three indicators: structural clarity, color contrast, and color distribution. Each indicator was assessed using a scoring system ranging from 1 to 3 on the observation sheet (Dafrita et al., 2020). The assessment was conducted by validators with expertise in microtechnique. The scores obtained from each treatment group, with four replications, were averaged and converted into percentages. The data were then statistically analyzed using the non-parametric Kruskal–Wallis test to determine differences among the eight treatment groups.

3. RESULTS AND DISCUSSION

Six treatments were tested for whole mount preparate quality against *Hydrocotyle umbellate* plants, which were then compared with the safranin control group and without staining. Based on the assessment of the three parameters of structural clarity, color contrast and color distribution, the percentage of preparation quality can be seen in Table 1.

Tabel 1. *Whole mount* preparation quality percentage

Parameter	Safranin	No Staining	t1 (70%, 15')	t2 (70%, 30')	t3 (80%, 15')	t4 (80%, 30')	t5 (90%, 15')	t6 (90%, 30')
Clarity of structure	96%	51%	75%	75%	85%	91%	68%	55%
Color contrast	93%	33%	78%	71%	76%	80%	75%	58%
Color distribution	76%	33%	80%	58%	83%	66%	58%	66%
%	**88%**	**39%**	**77%**	**68%**	**81%**	**79%**	**67%**	**59%**

Quality Percentage:
81-100 = Very good
61-80 = Good
41-60 = Fairly good
21-40 = Less good
0-20 = Not good

According to Table 1. in the structure clarity parameter, the percentage of preparation quality shows the difference between treatments. t4 shows the highest percentage of structure clarity quality in the excellent category, which is characterized by the identification of certain anatomical parts of *Hydrocotyle umbellata*. t5 shows the lowest percentage of structure clarity quality characterized by the non-identification of the anatomical part of the trichome and cellular components. Based on the follow-up post hoc test of the Kruskal-wallis test which showed significant differences ($p < 0.05$), in the structure clarity parameters, the results of the post hoc test

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showed that the concentration treatment was 90%, 30', concentration was 70%, 30', and concentration was 70%, 15' significantly different from the treatment without staining. Meanwhile, the treatment, including safranin and variations in concentration of 80% and 90% duration of 15', did not show significant differences with the other treatment groups.

The presence of percentage differences indicates that there is an effect of the treatment on the quality of the preparation of the structure clarity parameters. This is in line with previous research which stated that as the concentration of butterfly pea flower extract increases, the anthocyanin content also increases (Adrikayana & Pratiwi, 2022).

Increased anthocyanins can also affect the level of color contrast in the whole mount preparation of *Hydrocotyle umbellate* plants (Fenti Nurmailah et al., 2024). This can be seen through research data results which show that the higher the concentration of extract, the greater the color sharpness. t5 shows the highest color contrast percentage. However, too high a concentration of dye actually causes overstaining, so the anatomical structure will be difficult to distinguish. These findings are in line with previous research which stated that too high a concentration produces poor coloring which is caused by the concentration of pigment inhibiting color penetration in the cell wall (Atik et al., 2023).

In the color contrast parameter, significant differences based on the post hoc test were only found between the 70% concentration treatment of ,30' with the no-staining treatment and the 90%, 30' concentration. The other treatments showed relatively similar responses and did not differ statistically significantly.

The even distribution of color indicates optimal penetration of anthocyanin pigments, so that the tissue structure can be thoroughly observed (Rahmah & Ramdan, 2023). The most optimal color evenness is found in t3, which is characterized by the coloring results being evenly distributed so that there are no clumps or differences in color intensity in certain parts. However, at the same concentration with higher duration has the lowest percentage of color distribution. In the color distribution parameters, the duration of coloration did not show a significant effect.

Based on the results of the post hoc test of color distribution parameters, it shows that all treatments with notation b are significantly different from treatments without coloring and concentrations of 70% duration 30'. Meanwhile, there were no significant differences between treatments in the same group.

According to Laila et al., (2025) weak anthocyanin levels, pH, temperature, the presence of suboptimal anthocyanin absorption processes can inhibit the distribution of coloration in plant tissue. Not being precise in determining the position of the test plant during the staining process, where there are parts that are too flooded with dye, and there are also parts that are not flooded with dye, can cause less than optimal absorption of anthocyanins in stable plant tissue (Permatasari et al., 2023).

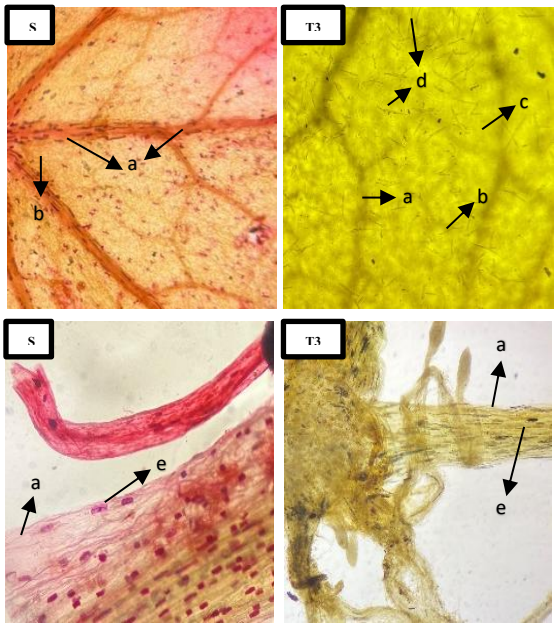


Figure 1. The appearance of a whole mount preparation of *Hydrocotyle umbellata* resulting from staining with T3 treatment and safranin dyeing of butterfly pea flower extract observed microscopically (40x microscope magnification). **(a)** leaf vein **(b)** vein branch **(c)** epidermal tissue **(d)** trichome **(e)** cellular components

4. CONCLUSION

The research results show that the use of dyes from natural ingredients in butterfly pea flower extract has an influence on the quality of Whole mount preparations. The best results were

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obtained in a combination of a concentration of 80% of the 15 minute staining duration expressed in the excellent category, and a concentration of 80% of the 30 minute staining duration expressed in the good category. Both combinations were closest to the percentage of preparation quality in the control treatment of safranin staining. As a suggestion for further research, further development needs to be carried out by setting the pH in the extraction process, considering that the stability and color yield of the extract is greatly influenced by pH conditions. Proper pH regulation is expected to produce a more optimal pigment color so that the coloring quality of whole mount preparations can be further improved.

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