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Effectivity of Rosella Flower Pressed Water (*Hibiscus sabdariffa* L.) as a Natural Coloring Agent for Examination of Feces Confirmed Helminthiasis

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Abstract

Background: Soil Transmitted Helminths (STH) are prevalent in tropics. Eosin 2% dye is commonly used for helminth egg identification, but this dye is carcinogenic and damaging to the environment.
Aim: This study evaluates the effectiveness of rosella pressed water (Hibiscus sabdariffa L.) as a natural dye for helminthiasis feces examination, examines its performance with added citric acid, and compares the staining results.
Methodology: This experimental study used a post-test only control group design with 27 purposively selected samples of 10% formalin-fixed helminthiasis feces. Staining was performed using rosella pressed water at concentrations of 10%, 20%, 30%, and 40%, with and without 9% citric acid, alongside 2% eosin as a comparison. Observations were made under a 10x microscope, and staining effectiveness was evaluated using a Likert scale by a parasitologist.
Results: The staining quality of rosella pressed water was consistent (median = 3) across all concentrations. Parasite visualization varied without citric acid, with reduced quality at 20% concentration (median = 2). Preparation quality remained stable (median = 3). Kruskal-Wallis analysis showed no significant differences ($p > 0.05$).
Discussion: Rosella flowers (*Hibiscus sabdariffa* L.) contain anthocyanin pigments which are flavonoid compounds that provide red to purple color, so they can be used as a natural dye for STH eggs.
Conclusion: This study showed that rosella flower pressed water was effective for STH egg staining, although 2% Eosin gave the best and most consistent results. There were no significant differences between treatments.

Keywords: : *Eosin 2%, Rosella flower pressed water, STH egg coloring, Citric acid*

Introduction

The World Health Organization (WHO) in 2019 reported that more than 1.5 billion people (24%) of the world's population are exposed to soil-transmitted helminths. Prevalence is highest in tropical and subtropical regions such as China, East Asia, sub-Saharan Africa, and the United States (Gulo & Marbun, 2024). Based on the results of surveys and research by the Ministry of Health of the Republic of Indonesia in 2018, in several provinces it was found that around 60% of the population had worm infections, with the largest age group aged 5-14 years. Of the 60% prevalence rate, 21% of them attacked children with an average number of worms per person of six. The highest prevalence was found in West Nusa Tenggara, West Sumatra and North Sumatra provinces. Helminths are found in areas with high humidity, especially in communities with poor personal hygiene and environmental sanitation (Cakrawati et al., 2024).

Soil Transmitted Helminth (STH) can be identified by laboratory examination which aims to identify the egg stage (Ningsih et al., 2023). In examining worm eggs, there are several methods that can be used, such as direct examination with the native method (direct slide), sedimentation, Kato Katz, and flotation. In fecal examination by the native method, staining is required to facilitate identification of the worm egg shape, clarify the appearance, and increase background contrast in preparations observed with a microscope. 2% eosin stain is considered the gold standard and is often used in staining for qualitative examination due to its sensitivity, ease of use, and rapid results (Dako et al., 2024).

Research by Jumardi, et al. (2023) the use of eosin has carcinogenic properties, carcinogenic properties that are listed as class 3 IARC carcinogens if used continuously, because they contain chemicals including picric acid, chloric hydrate. The use of eosin causes negative effects, such as irritation to the eyes, skin, mucous membranes and causes cancer for medical laboratory technologists who are frequently exposed. The remaining waste can also be harmful to the

environment. Therefore, alternative dyes are needed to reduce the impact of eosin use, one of which is by using natural dyes (Dako et al., 2024).

The utilization of one of the flora that can be used as a natural dye substitute for eosin, namely rosella flowers (*Hibiscus sabdariffa* L.) can be a source of natural red dye because this flower contains anthocyanins which belong to the flavonoid group. Apart from being a dye, rosella flowers (*Hibiscus sabdariffa* L.) are one of the plants that can be used as a preservative, because they contain antioxidants and antibacterials (Nurcahyo&Kusnadi, 2019). Anthocyanins are a class of organic chemical compounds that can dissolve in polar solvents and are responsible for providing color in plants (Priska et al., 2018). Based on the results of previous research on the use of rosella flowers as a coloring, good results were obtained in coloring Soil Transmitted Helminth (STH) eggs using rosella flower baths with a concentration of 1: 1 dilution of aquadest and 1: 2 dilution, but the staining resistance test has not been studied (Laandrise, 2021).

Currently, there is no research data regarding the application of rosella flower pressed water combined with organic acid, specifically citric acid, for staining feces confirmed to contain helminths. Thus, the researchers were compelled to do a study entitled: "Effectivity of Rosella Flower (*Hibiscus sabdariffa* L.) Pressed Water as a Natural Coloring Agent for the Examination of Feces Confirmed Helminthiasis."

Method

The research design used a true experimental design with a post-test only control group design. The research was conducted at the Pharmacology and Clinical Pharmacy Laboratory, Faculty of Pharmacy, Universitas Muhammadiyah Surakarta, to prepare rosella flower pressed water at concentrations of 10%, 20%, 30%, and 40%, with or without 9% citric acid. The effectiveness of the rosella flower pressed water was tested as a feces dye on confirmed helminthiasis feces samples at the Parasitology Laboratory of FK-KMK Universitas Gadjah Mada. The research was carried out in November 2024. Ethical clearance letter was issued by the Health Research Ethics Commission of Dr. Moewardi Hospital with Number: 2.575/X 1 HREC 1 2024. Rosella flowers were taken from Tawangmangu, Karanganyar. Determination of plant species was carried out at the Testing Laboratory - UPF Traditional Health Services of Dr. Sardjito Hospital, Tawangmangu, with the number TL.02.04/D.XI.6/22566.1089/2024. The determination results showed that the plant species was *Hibiscus sabdariffa* L., with the synonym *Sabdariffa rubra* Kostel.

The research population consisted of confirmed helminthiasis feces samples obtained from the Parasitology Laboratory of FK-KMK Universitas Muhammadiyah Surakarta. This research used helminthiasis feces samples preserved in 10% formalin. Sampling was conducted using a purposive sampling technique. The sample size estimation in this study was calculated using the Federer formula, resulting in three repetitions consisting of nine groups with a total of 27 samples. The independent variable in this study was rosella flower pressed water at concentrations (10%, 20%, 30%, 40%) with or without the addition of 9% citric acid. The dependent variables included staining effectiveness (intensity, stability, clarity), visualization of parasite structures (clarity, contrast), and the quality of staining results (uniformity). Inclusion criteria included feces samples confirmed for helminthiasis in good condition and preserved with 10% formalin, while exclusion criteria included feces samples in the form of finished preparations.

The preparation of rosella flower pressed water with or without the addition of 9% citric acid began by selecting fresh and clean rosella flowers. Then, the rosella petals were separated, and the seeds were discarded. The rosella flowers were washed under running water to remove any dirt or dust and dried by patting them with a clean cloth or tissue. The cleaned rosella flowers were placed in a juicer and pressed to obtain the juice, which was collected in a beaker glass. The pressed water was filtered to separate the pulp using a sieve or filter paper. After filtration, distilled water was added to make up to 100 mL, with or without the addition of 9% citric acid (900 mg per 100 mL solution) according to the predetermined concentrations. The rosella flower pressed water was stored in a black bottle and refrigerated to maintain freshness.

The research process began with cleaning an object glass and applying a small amount of confirmed helminthiasis feces sample (± 2 mg) using the tip of a stick. One to two drops of rosella flower pressed water (with or without citric acid) and 2% Eosin were added to the object glass and mixed until homogeneous. Coarse particles, if any, were removed. A deck glass was used to cover the preparation without trapping air bubbles. Observations were made under a microscope at 10x magnification, and the findings were documented.

Staining evaluation included assessing staining effectiveness, visualization of parasite structures, and the quality of the preparation by several experts in parasitology using the Likert scale (scale 1-3) (Sophia & Km, 2022). For the criteria to assess staining effectiveness, researchers assigned scores of 1, 2, and 3, referring to the study by (Oktari and Mutamir, 2017) as follows:

Table 1. Assessment scores (Likert scale)

Score	Descriptions
Score (1)	If the field of view is not contrasted, STH eggs do not absorb the dye, and egg parts are not visible.
Score (2)	If the field of view is less contrasted, STH eggs absorb less dye, and egg parts are less visible
Score (3)	If the field of view is contrasted, STH eggs absorb the dye, and egg parts are visible.

Statistical analysis used Jefferys's Amazing Statistics Program (JASP) statistical software at a significance level of 0.05. Starting with a normality test to determine data distribution using the Shapiro-Wilk test. Then, the data underwent the Levene test to determine homogeneity. If the data were normally distributed and homogeneous, parametric testing continued with One-Way ANOVA and Post Hoc LSD. If the data were not normally distributed or homogeneous, the Kruskal-Wallis parametric test was used, followed by Post Hoc Mann-Whitney.

Results and Discussion

Rosella flower obtained from Tawangmangu, Karanganyar, were determined at the Testing Laboratory-UPF Traditional Health Services of Dr. Sardjito Hospital, Tawangmangu. The results of sugarcane plant determination are shown in table 2.

Table 2. Determination Results of Rosella Flower

Taxonomy	Results
Family	Malvaceae
Species	*Hibiscus sabdariffa* L.
Synonyms	*Sabdariffa rubra* Kostel.

Figure 1. Rosella Flower (*Hibiscus sabdariffa* L.) plants obtained from Tawangmangu, Karanganyar

Organoleptic test is a method used to evaluate the physical properties of a product through the five human senses, such as taste, smell, color, texture, and appearance. This test aims to provide an initial introduction to the physical properties of rosella flower juice using the five senses (Sahu et, al. 2020).

The results of the organoleptic test of rosella flower pressed water obtained a dark purplish-red, with a consistency like water. The pressed water looks a little cloudy, possibly due to anthocyanin pigments from rosella petals. A total of 500 mL of rosella flower pressed water was obtained from 3 kg of fresh rosella flowers. This pressed water has a fresh sour aroma (Namira & Hendradi, 2023).

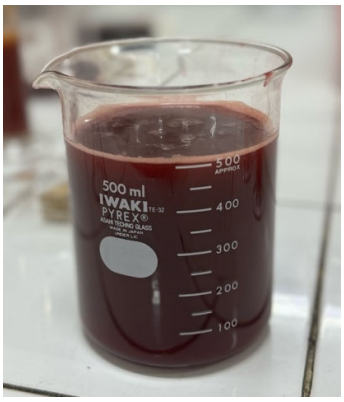


Figure 2. Organoleptic test Rosella Flower (*Hibiscus sabdariffa* L.) pressed water

The pH test using litmus paper is a simple method to determine the acidity or basicity of a solution. Litmus paper comes in two colors: red and blue. Red litmus paper will turn blue when exposed to a basic solution, while blue litmus paper will turn red when exposed to an acidic solution. This color change indicates the acidic or basic nature of the solution being tested (Wibowo, 2019).

The results of the pH test obtained rosella flower pressed water has a pH of 1 for eight concentrations, indicating its acidic nature. This is due to the content of organic acids, such as citric acid and malic acid, which are naturally present in rosella petals (Tensiska et al., 2017)

Figure 3. pH test Rosella Flower (*Hibiscus sabdariffa* L.) pressed water

The stability test aims to evaluate the durability and changes that may occur in rosella flower juice during storage. It includes observations on physical stability and pH. This test involves observations over a period of time under specific storage conditions (Monita et, al. 2023).

The stability test of rosella flower pressed water conducted by storing it in a refrigerator at about 5°C and observing whether there were any physical changes, showed that the solution remained stable and durable. Storage at low temperatures helps slow down the degradation process of anthocyanin pigments, which are the main coloring agents in rosella flowers (Amperawati et al., 2019).

At low temperatures, microbial activity and chemical reactions that can damage the solution are slower, so the solution remains clear and shows no signs of deterioration, such as significant changes in color, odor, or sediment. This proves that cold storage is effective for maintaining the quality and stability of rosella flower juice for a long period of time (Sari et al., 2023).

Figure 4. Stability test Rosella Flower (*Hibiscus sabdariffa* L.) pressed water

Observations were made by 3 panelists/verifiers who are experts in Parasitology. The microscopic features of the worm eggs were as follows:

Table 3. Microscope observation results

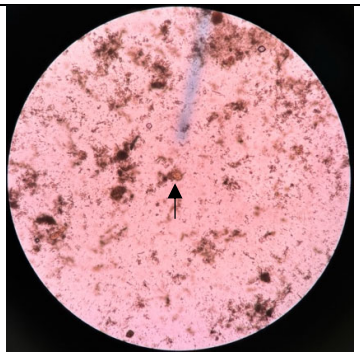
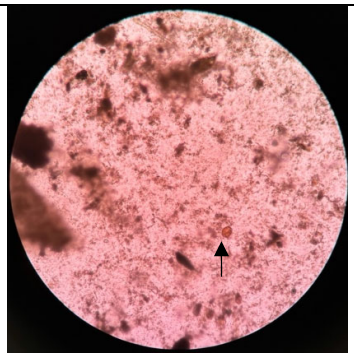
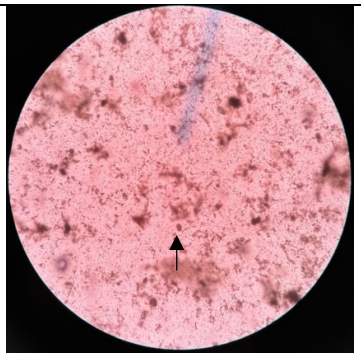
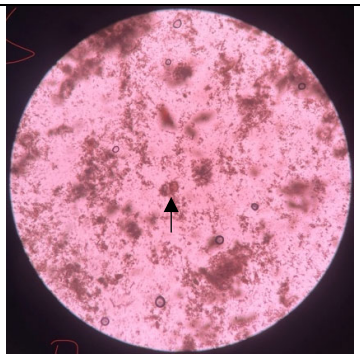
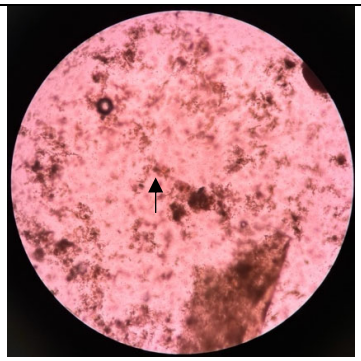
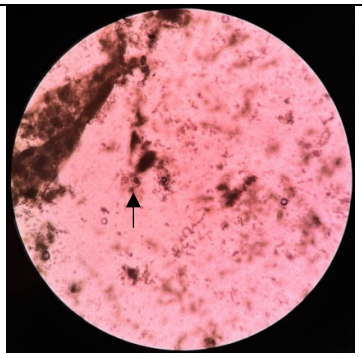
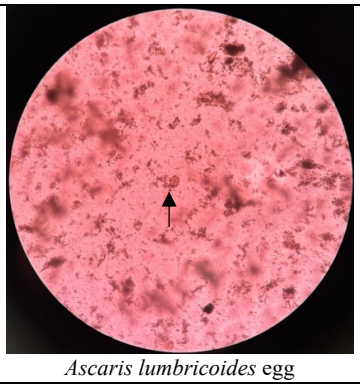
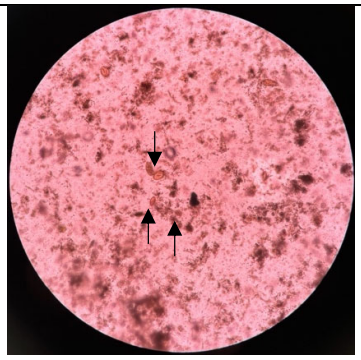
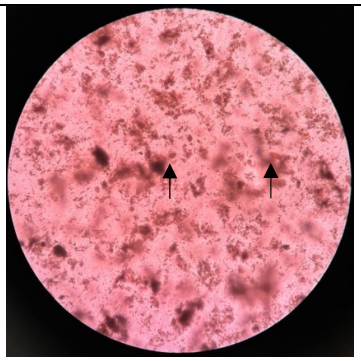
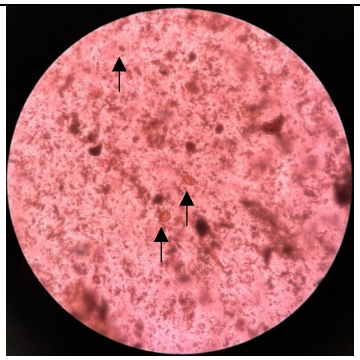
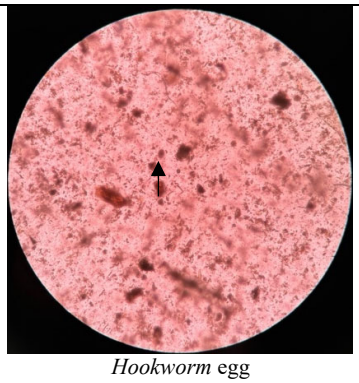
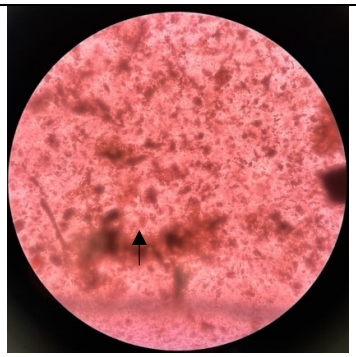
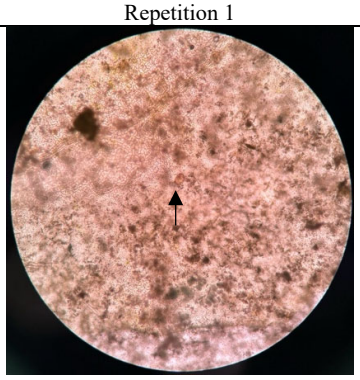
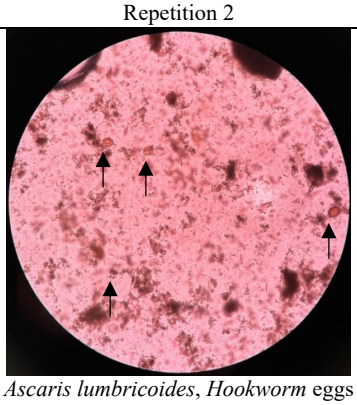
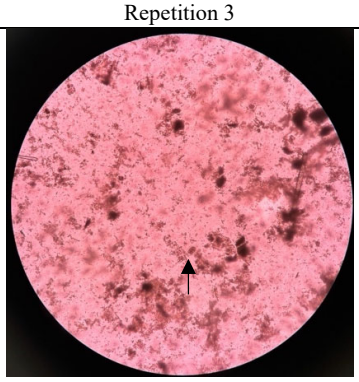
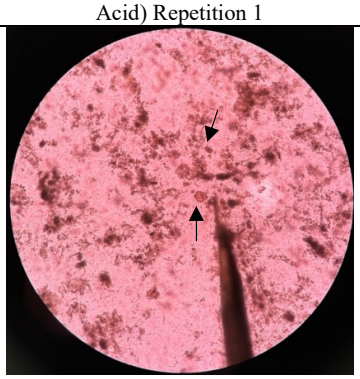
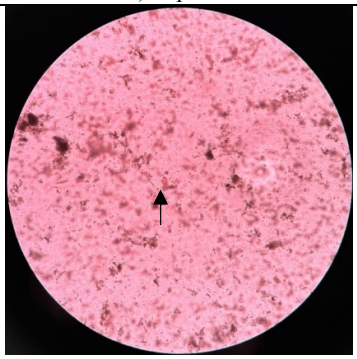
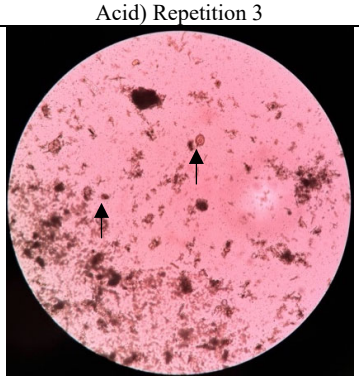
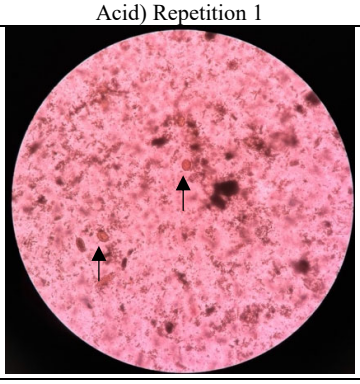
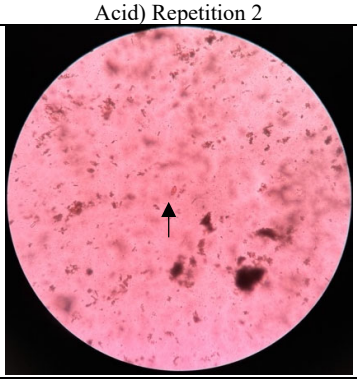
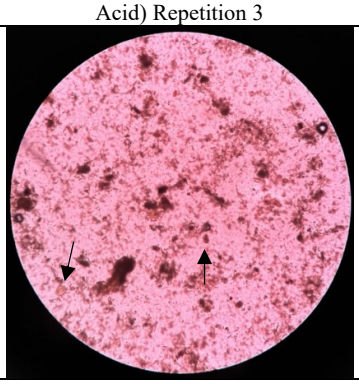
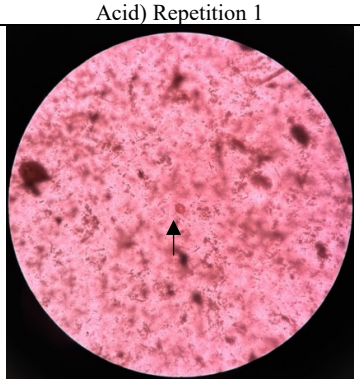
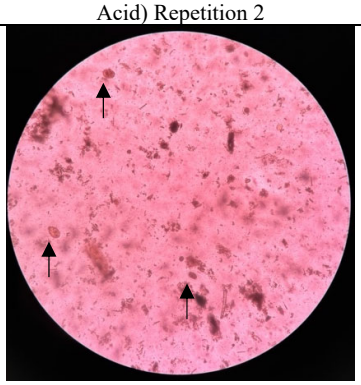
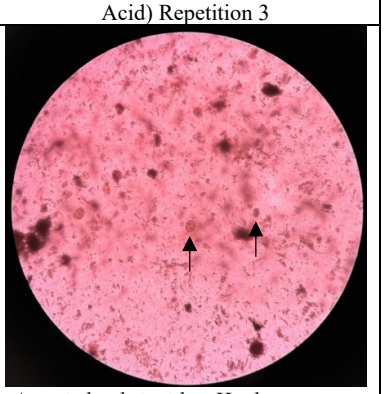
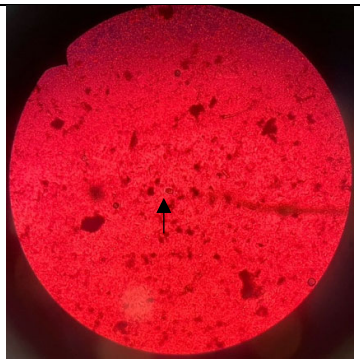
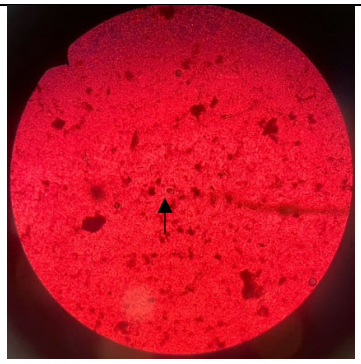
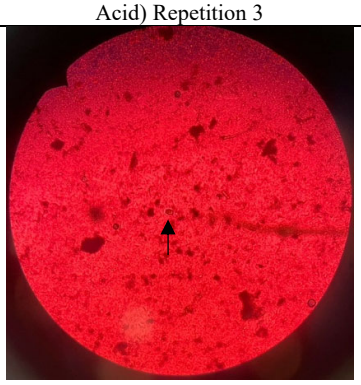
 *Ascaris lumbricoides* egg Figure 1. 10% concentration Repetition 1	 *Ascaris lumbricoides* egg Figure 2. 10% concentration Repetition 2	 *Hookworm* egg Figure 3. 10% concentration Repetition 3
 *Ascaris lumbricoides* egg Figure 4. 20% concentration Repetition 1	 *Ascaris lumbricoides* egg Figure 5. 20% concentration Repetition 2	 *Ascaris lumbricoides* egg Figure 6. 20% concentration Repetition 3
 *Ascaris lumbricoides* egg		 *Hookworm*, *Trichuris trichiura* eggs

Figure 7. 30% concentration Repetition 1	*Ascaris lumbricoides*, *Hookworm*, *Trichuris trichiura* eggs Figure 8. 30% concentration Repetition 2	Figure 9. 30% concentration Repetition 3
 *Ascaris lumbricoides*, *Hookworm* eggs Figure 10. 40% concentration Repetition 1	 *Hookworm* egg Figure 11. 40% concentration Repetition 2	 *Ascaris lumbricoides* egg Figure 12. 40% concentration Repetition 3
 *Ascaris lumbricoides*, *Hookworm* eggs Figure 13. 10% Concentration (Citric Acid) Repetition 1	 *Ascaris lumbricoides*, *Hookworm* eggs Figure 14. 10% Concentration (Citric Acid) Repetition 2	 *Hookworm* egg Figure 15. 10% Concentration (Citric Acid) Repetition 3
 *Ascaris lumbricoides*, *Hookworm* eggs Figure 16. 20% Concentration (Citric Acid) Repetition 1	 *Trichuris trichiura* egg Figure 17. 20% Concentration (Citric Acid) Repetition 2	 *Ascaris lumbricoides*, *Hookworm* eggs Figure 18. 20% Concentration (Citric Acid) Repetition 3
		

Ascaris lumbricoides, *Hookworm* eggs Figure 19. 30% Concentration (Citric Acid) Repetition 1	*Trichuris trichiura* egg Figure 20. 30% Concentration (Citric Acid) Repetition 2	*Ascaris lumbricoides, Hookworm* eggs Figure 21. 30% Concentration (Citric Acid) Repetition 3
 *Ascaris lumbricoides* egg Figure 22. 40% Concentration (Citric Acid) Repetition 1	 *Ascaris lumbricoides*, *Hookworm* eggs Figure 23. 40% Concentration (Citric Acid) Repeat 2	 *Ascaris lumbricoides*, *Hookworm* eggs Figure 24. 40% Concentration (Citric Acid) Repetition 3
 *Hookworm* egg Figure 25. with 2% Eosin as a control	 *Hookworm* egg Figure 26. with 2% Eosin as a control	 *Hookworm* egg Figure 27. with 2% Eosin as a control

Rosella flowers (*Hibiscus sabdariffa* L.) can be used for worm egg coloring because of the anthocyanin pigment contained in the petals. Anthocyanins are flavonoid compounds that give rosella flowers a red to purple color and function as natural coloring agents (Djaeni., et al 2017). In this study, worm egg staining aims to facilitate the study of the shape of worm eggs in feces suspensions confirmed helminthiasis, clarify and see the shape of worm eggs, and contrast in worm egg preparations using a microscope. Staining using 2% Eosin produces a red color in the cytoplasm, contrast field of view and worm eggs absorb color. The higher the score, the better the quality of the staining, the contrast with the field of view, the worm eggs are colored and the egg parts are clearly visible (Apriani & Ereskadi, 2022).

The treatment using rosella flower pressed water at concentrations of 10%, 20%, 30%, 40% with or without 9% citric acid provides good staining effectiveness, visualization of parasite structures, and quality of staining results. However, there are several treatments that have a score of 2, at concentrations of 10%, 20%, 30% in the third repetition on the visualization of parasite structures, as well as at a concentration of 10% with the addition of 9% citric acid in the first repetition, which means that it has a less contrasting field of view, worm eggs absorb less color and parts of the worm eggs are less clearly visible. Eosin 2% as a comparison control produces a score value of 3 which is the highest score, meaning that the quality of staining with Eosin 2% provides the best staining quality, meaning that it has a contrasting field of view, worm eggs absorb color and parts of the eggs are clearly visible.

In this study there were several limitations experienced by researchers, namely the results of rosella flower pressed water could not be durable at room temperature and the preparation was found to dry out (approximately within 1 hour), because rosella flower pressed was added with pure distilled water which consisted of water without any dissolved substances such as salts or minerals and distilled water experienced natural evaporation (Khotimah et al., 2018). The addition of 9% citric acid also gave the same effect.

Table 4. Descriptive Analysis of Rosella Flower Pressed Water

Concentration	Median (IQR)		
	Effectiveness of Coloration	Visualization of Parasite Structure	Quality of Coloring Results
10%	3 (0,500)	3 (0,500)	3 (0,000)
20%	3 (0,000)	2 (0,500)	3 (0,000)
30%	3 (0,000)	3 (0,500)	3 (0,000)
40%	3 (0,000)	3 (0,000)	3 (0,000)
Eosin 2%	3 (0,000)	3 (0,000)	3 (0,000)

Table 4. Descriptive Analysis of Rosella Flower Pressed Water

Data from table 3, descriptive analysis for rosella flower pressed water based on concentration on staining effectiveness, visualization of parasite structure, and quality of staining results. All results are reported in the form of Median (IQR), the median provides information about the center of the data distribution, while the IQR provides an overview of the distribution of data around the median.

Staining Effectiveness. Median = 3 for all concentrations (10%, 20%, 30%, 40%) and 2% eosin. IQR = 0.500 only at 10% concentration, indicating little variation between samples. The rest of the concentrations show IQR = 0, which means the data is uniform. All concentrations had the same median value (3), indicating that the staining effectiveness was relatively good and consistent across all concentrations. The 10% concentration showed little variation compared to the other concentrations.

Visualization of Parasite Structure. Median = 3 for concentrations of 10%, 30%, 40%, and 2% eosin. However, for the 20% concentration, the median dropped to 2. IQR = 0.500 at 10%, 20%, and 30% concentrations, indicating variation between samples at these concentrations. The 40% concentration and 2% eosin had an IQR = 0 (uniform data). The 20% concentration had a lower median (2), indicating slightly poorer results in visualization of parasite structure than the other concentrations. Variation between samples was seen at 10%, 20%, and 30% concentrations (IQR = 0.500).

Quality of Staining Results. Median = 3 for all concentrations (10%, 20%, 30%, 40%) and 2% eosin. IQR = 0.000 at all concentrations, indicating that the data is highly uniform with no variation. All concentrations showed consistent staining results (median = 3, IQR = 0).

Table 5. Descriptive Analysis of Rosella Flower Pressed Water with 9% Citric Acid

Concentration	Median (IQR)		
	Effectiveness of Coloration	Visualization of Parasite Structure	Quality of Coloring Results
10% + Citric Acid	3 (0,000)	3 (0,500)	3 (0,000)
20% + Citric Acid	3 (0,000)	3 (0,500)	3 (0,500)
30% + Citric Acid	3 (0,000)	3 (0,500)	3 (0,000)
40% + Citric Acid	3 (0,000)	3 (0,000)	3 (0,000)
Eosin 2%	3 (0,000)	3 (0,000)	3 (0,000)

Table 5. Descriptive Analysis of Rosella Flower Pressed Water with 9% Citric Acid

Data from table 4, descriptive analysis for rosella flower pressed water with the addition of 9% citric acid at various concentrations on the effectiveness of staining, visualization of parasite structure, and quality of staining results. All results are reported as Median (IQR), the median provides information about the center of the data distribution, while the IQR provides an overview of the spread of data around the median.

Staining Effectiveness. Median = 3 for all concentrations (10% + citric acid, 20% + citric acid, 30% + citric acid, 40% + citric acid) and 2% eosin. IQR = 0.000 for all concentrations, indicating that the data is highly uniform with no variation. All concentrations gave uniform results (median = 3, IQR = 0.000). The addition of citric acid did not alter the effectiveness of the staining.

Visualization of Parasite Structure. Median = 3 for all concentrations, indicating good and consistent performance in parasite structure visualization. IQR = 0.500 at 10%, 20%, and 30% concentrations, indicating little variation between samples at these concentrations. The 40% concentration and 2% eosin had IQR = 0 (uniform data). All concentrations produced the same median (3), indicating good performance. Slight variations were seen in the 10%, 20%, and 30% concentrations (IQR = 0.500), while the 40% concentration and 2% eosin showed uniform data.

Quality of Staining Results. Median = 3 for all concentrations (10% + citric acid, 20% + citric acid, 30% + citric acid, 40% + citric acid) and 2% eosin. IQR = 0.000 at 10%, 30%, 40%, and 2% eosin concentrations, indicating that the data were uniform without variation. At 20% concentration, the IQR = 0.500, indicating slight variation between samples. All concentrations had the same median (3), indicating good staining quality. Slight variation occurred at 20% concentration (IQR = 0.500), while the other concentrations were uniform.

Table 6. Bivariate Analysis with Kruskal Wallis Test

Variable	P-value
Effectiveness of Coloration	P 0,433
Visualization of Parasite Structure	P 0,654
Quality of Coloring Results	P 0,433

Table 6. Bivariate Analysis with Kruskal Wallis Test

Data from table 5, bivariate data analysis with Kruskal Wallis test for non-parametric test used to compare the distribution of data in more than two independent groups. The p-value indicates whether there is a statistically significant difference between the groups. Analysis results obtained staining effectiveness p=0.433, visualization of parasite structure p=0.654, quality of staining results: p=0.433 which means the p-value for all variables is greater than 0.05. Thus, there were no statistically significant differences between the groups for all variables tested.

Conclusions

This study shows that staining using rosella flower pressed water with various concentrations (10%, 20%, 30%, 40%) with or without the addition of 9% citric acid has good effectiveness in visualizing parasite structures and staining quality, although there is little variation in some concentrations. Staining with 2% Eosin as a control gave the best results with the most consistent values and optimal quality. Statistical analysis showed no significant differences between the treatment groups. However, rosella flower pressed water has limitations in solution durability at room temperature, although storage at low temperature (5°C) can improve this. Overall, a 40% concentration of rosella flowers produced good staining, but 2% Eosin remained the most superior method.

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