Indonesian Journal of Medical Laboratory Science and Technology
Open Access
p-ISSN 2684-6748
e-ISSN 2656-9825

RESEARCH ARTICLE

The comparison of the effectiveness of shoe flower (*Hibiscus rosa-sinensis* L.) and roselle flower (*Hibiscus sabdariffa* L.) infusions as alternative reagents for the examination of Soil Transmitted Helminths (STH) eggs

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Article history:
Received: 2023-11-29
Revised: 2024-02-19
Accepted: 2024-03-18
Avalibale online: 2024-04-29

Keywords:
Soil transmitted helmints
Infusions
Hibiscus rosa-sinesis L.
Hibiscus sabdariffa L.
Egg examination

https://doi.org/10.33086/ijmlst.v6i1.5409

Abstract

Worm infections remain a significant health challenge globally, including in Indonesia, where they continue to contribute to a majority of health issues. Soil-transmitted helminths (STH), particularly intestinal nematode parasites, are predominant in causing these infections. Microscopic examination with 2% eosin staining is a conventional method for diagnosing worm infections. However, exploring alternative staining agents, such as anthocyanin-rich extracts from *Hibiscus rosa-sinensis* L. (hibiscus flower) and *Hibiscus sabdariffa* L. (roselle flower), presents a promising avenue. These plants possess anthocyanin compounds, resulting in a captivating red pigment and making them suitable for utilization as natural coloring agents. This study aimed to evaluate the efficacy of hibiscus and roselle infusions as alternative staining reagents for examining STH egg, compared to the standard 2% eosin staining method. Adopting a true experimental design, samples of STH worm egg suspensions were subjected to different concentrations of hibiscus and roselle infusions. The results revealed varying staining outcomes with different concentrations. However, a 1:1 concentration ratio of hibiscus and roselle infusions demonstrated effective staining against STH worm eggs. In conclusion, this research underscores the comparative effectiveness of hibiscus and roselle infusions as alternative staining reagents for STH egg examination, with 2% eosin serving as the control. These findings hold potential implications for improving diagnostic techniques in managing worm infections.

1. INTRODUCTION

One type of disease that is still common in Indonesia among the people caused by parasitic worm infection of the group of *soil-transmitted helminths* (STH). STH are a group of worm parasites whose life cycle is transmitted through the soil. There are four types of worm species belonging to the STH group which cause health problems, namely *Ascaris lumbricoides*, *Trichuris trichiura*, *Strongyloides stecoralis*, *Necator americanus* and also *Ancylostoma* sp (1). In 2020, the *World Health Organization* (WHO) stated that the incidence of worms in the world is relatively high. Specifically, more than 1.5 billion people or 24% of the world's population have been infected by the parasitic type of STH (2). The prevalence of worm infection in Indonesia is relatively high, especially in densely populated environments with poor sanitation. In Indonesia, nearly 200 million people across 31 provinces are estimated to be at risk of STH infection. Participants were positive for STH with prevalences of 26%, 7.9% and 1.8% identified for *A. lumbricoides*, hookworm and *T. trichiura* at 7.9% and 1.8%, respectively (3). The prevalence of worms was recorded at 30-90% of children under 12 years old. This number shows that most worm

Citation: Charisma AM, Rahayu EO, Arianing IF. The comparison of the effectiveness of shoe flower (Hibiscus rosa-sinesis L.) and roselle flower (Hibiscus sabdariffa L.) infusions as alternative reagents for the examination of Soil Transmitted Helminths (STH) eggs. Indones J Med Lab Sci Technol. 2024;6(1):65–74. https://doi.org/10.33086/ijmlst.v6i1.5409

infections are mostly found in children. From environmental survey data, this found that the situation is sufficiently conducive to the worm's life cycle (4).

Indeed, staining methods utilizing eosin dye offer a reliable means for examining worm parasite eggs. Eosin dye imparts a red coloration to the background, providing a stark contrast against the yellow hue of the eggs. This stark color contrast facilitates the precise identification and differentiation of eggs from fecal matter, ensuring accuracy in the examination process (5). Worm egg examination using 2% eosin reagent can harm the environment and water sources because they prefer to decompose and eventually become toxic (6). For this reason, an alternative colouring method that is more eco-friendly is needed, namely natural dyes from nature. Apart from eosin dye, there are other dyes that are thought to colour worm eggs, namely anthocyanin (7).

The hibiscus flower (*Hibiscus rosa-sinensis* L.) is one of the natural plant used as a dye, which has eosin-like traits. *Hibiscus sabdariffa* flowers have been identified as having active compounds such as flavonoids, saponins, anthocyanins and other substances that can be used as natural dyes and preservatives. Anthocyanins contain natural dye that shows discolouration response to pH variations over a wide range (8). Several plants containing anthocyanins are used as natural dyes. Anthocyanins can be found in various plants, especially in flowers. For example, in Roselle flowers, hibiscus flowers, and roses *(Rosa damascena*). They are used as dyes for type L. donovani parasites identification (9). They have properties at specific pH levels that affect the colour of anthocyanins. At acidic pH, anthocyanins will be red or purple. Although, it will turn green or yellow at alkaline pH and blue at neutral pH. Therefore, anthocyanin compounds are usually used as colour indicators and natural dyes (10).

Anthocyanin is fully coloured in green plants from its flowers, fruit and leaves. Moreover, it is used as a natural colouring in various food products and applications. For flowers, the anthocyanin dye from hibiscus flowers can arise due to the long conjugated double bonds that can absorb all light, making anthocyanin an antioxidant with a radical scavenging mechanism (11). Some anthocyanin compounds most commonly found in hibiscus flowers are pelargonidin, peonidin, cyanidin, malvidin, petunidin, and delphinidin. Roselle flowers contain several secondary metabolic compounds. The most dominant in red rosella flowers is the presence of anthocyanin compounds, which form flavonoids and act as antioxidants (12). The flavonoids in rosella flowers consist of flavonols and anthocyanin pigments. Anthocyanin function as an antioxidant which is believed to cure degenerative diseases and produces a red colour which is used as a natural dye for making food ingredients and other alternative colourings (13).

A research showed that the hibiscus flower as an alternative colouring for parasites, fungi, and neural tissues (14). In other research (15), rosella flower also became an alternative colouring examining *soil-transmitted eggs* with concentrations of 80% and stable within 21 days of storage in cold temperatures. Therefore, researchers are interested in conducting comparative research on the effectiveness of soaking hibiscus and rosella flowers as alternative reagents for examining soil-transmitted helminths (STH) eggs with 2% eosin as a control.

2. MATERIALS AND METHODS

2.1. Study Design

This type of research is a true experimental with a completely randomized design (CRD) post-test control and received ethical approval from the Health Research Ethics Commission (KEPK) Airlangga University Faculty of Dental Medicine Health with ethical number 725/HRECC.FODM/VI/2023.

2.2. Collection of Clinical Data

In this research, a total of 20 treatments were utilized, and the study was conducted over a duration of one month at the Integrated Biology Laboratory, Anwar Medika University. The soil-transmitted helminth egg suspensions used for sampling were obtained from the Health Laboratory Center (BBLK) Surabaya, Indonesia.

2.3. Materials

The tools and materials used in this study were 50 mL brown bottles, hot plates, filter tool, iron stirrer, analytical balance, beaker glass, object glass, cover glass, pipette, microscope, hibiscus flower, rosella flower, label, suspension worm eggs, 2% eosin, tissue, water infusions in hibiscus and rosella flowers with varying concentrations of 1:1, 1:2, 1:3, and 1:4.

2.4. Observing Worm Eggs Is The Direct Method Using Water Infusions In Hibiscus and Roselle Flowers

The research method used in this study is the immersion method of hibiscus and roselle flowers. Hibiscus and rosella flowers, which have been weighed as much as 100 grams, are then soaked respectively in heated distilled water as much as 10 mL, 20 mL, 30 mL, and 40 mL for 24 hours after that the soaking is filtered and put into a brown bottle, which has been given a label. Stool examination was carried out using a direct method using dye infusions in hibiscus and roselle flowers in concentrations of 1:1, 1:2, 1:3, and 1:4 and 2% eosin as a control. The procedure for observing worm eggs is the direct method using water infusions in rosella flowers with a concentration of 1:1, 1:2, 1:3, and 1:4 and 2% eosin as a control. A glass object is selected and cleaned thoroughly to ensure it is free from any residue of fat. Then, a drop of worm egg suspension and another drop of water infused with rosella flowers, both at a concentration ration of 1:1, are place onto the glass. he mixture is then homogenized and covered with another glass cover, ensuring even distribution to prevent the formation of air bubbles. Next, the glass was observed under a microscope using 10x to 40x magnification and then photographed using an optilab. Observations were made using variations in the concentration of rosella flower infusions water of 1:1, 1:2, 1:3, and 1:4 and 2% eosin as a control.

2.5. Statistical Analysis

This research used Kruskal-Wallis and Man-U Withney hypothesis testing methods. For the criteria for assessing the effectiveness of research test results using each plant's infusion water, scores 1 to 3 were given. A score of 1 is given if the visual field does not have contrast, the worm eggs do not absorb colour and the worm eggs are not certainly visible, a score of 2 is given if the visual field lacks contrast, the worm eggs absorb fewer colour, and the egg is not certainly visible, and a score of 3 is given if the contrasting visual field of the worm egg absorbs the colour, and the worm egg is certainly visible.

3. RESULTS AND DISCUSSION

3.1. Comparative Research on The Effectiveness of Hibiscus and Roselle Flower Infusions As Alternative Reagents in Examining Soil Transmitted Helminths (STH) Eggs

In the comparative research evaluating the effectiveness of hibiscus and roselle flower infusions as alternative staining agents for examining soil-transmitted helminths (STH) eggs, the staining assessments were conducted using hibiscus and roselle flower infusions at various concentrations, alongside 2% eosin (control). These results are summarized in Table 1.

Table 1 . Results of infused hibiscus and roselle flowers with 2% eosin as a control

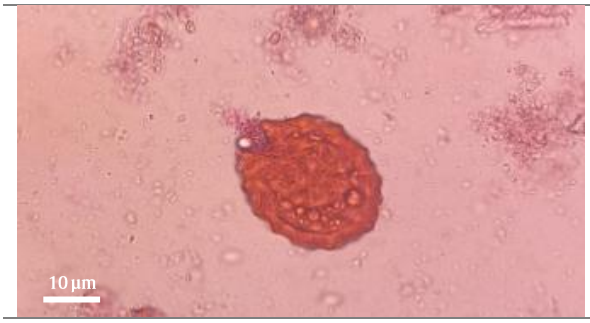
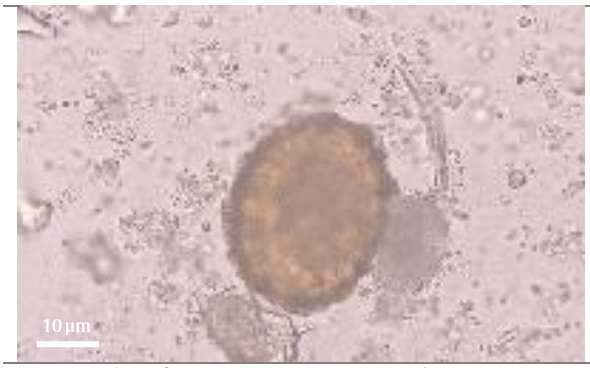
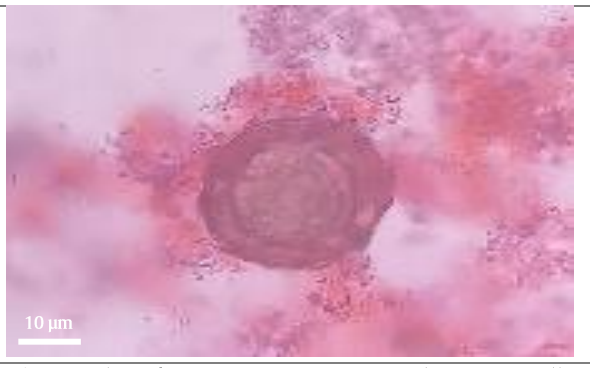
Dye type	Concentration	Coloring quality assessment results								
		Ascaris lumbricoides			*Trichuris trichiura*			*Hookworms*		
		1	2	3	1	2	3	1	2	3
Eosin 2% (control)	2%	0	100	0	0	100	0	0	100	0
	2%	0	100	0	0	100	0	0	100	0
	2%	0	100	0	0	100	0	0	100	0
	2%	0	100	0	0	100	0	0	100	0
Infused Hibiscus flowers	1:1	0	100	0	25	75	0	0	100	0
	1:2	25	75	0	0	100	0	25	75	0
	1:3	50	50	0	25	75	0	0	100	0
	1:4	50	50	0	0	100	0	50	50	0
Infused Roselle flowers	1:1	0	100	0	0	100	0	0	100	0
	1:2	25	75	0	0	100	0	0	100	0
	1:3	50	50	0	0	100	0	50	50	0
	1:4	50	50	0	0	100	0	50	50	0

Based on Table 1, the results using 2% eosin dye as a control showed 100% good results for *A. lumbricoides*, *T. trichiura* and *hookworm* eggs. When staining using hibiscus flower infusions with a concentration of 1:1, the results were 100% favourable for *A. lumbricoides* eggs, while for *T. trichiura* the results were 25% unfavorable and 75% unfavourable and hookworm eggs the results were 100% unfavourable. When staining using hibiscus flower infusions with 1:2 concentration, the results were 25% unfavourable and 75% unfavourable for *A. lumbricoides*

eggs, for *T. trichiura* eggs the results were 100% unfavourable, while for *hookworm* eggs the results were 25% unfavorable and 75% not good. When staining using hibiscus flower infusions and the soaking concentration was 1:3, the results were 50% bad for *A. lumbricoides* eggs, for *T. trichiura* eggs the results were 25% and 75% bad, while for *hookworm* eggs the results were 100% not good. When staining using hibiscus flower infusions, the infusions concentration was 1:4, the results were 50% and 50% not good on *A. lumbricoides* eggs, on *T. trichiura* eggs the results were 100% not good, while on hookworm eggs the results were 50 % not good. Based on the assessment results, the hibiscus flower infusion concentration that has the best quality is close to 2% eosin in colouring STH eggs is the infused hibiscus flower concentration 1:1.

In staining using roselle flower immersion concentration of 1:1, 100% yield was not good on *A. lumbricoides*, *T. trichiura*, and *hookworm* eggs. In staining using roselle flower immersion concentration of 1:2, 25% and 75% was not good for *A. lumbricoides* eggs, 100% bad results were obtained for *T. trichiura* eggs, while 100% bad results were obtained for hookworm eggs. In staining using rosella flower infusions concentrations of 1:3 and 1:4, 50% yield and 50% was not good for *A. lumbricoides* eggs, for *T. trichiura* eggs, 100% yield was not good, whereas for *hookworm* eggs, 50% yield was obtained not good. Based on the assessment results, the concentration of hibiscus infusions, which has the best quality, close to 2% eosin in coloring *soil transmitted helminths* eggs is hibiscus infusions concentration of 1:1. Images depicting the assessment of coloring results using soaked hibiscus and rosella flowers are presented in the Table 2.

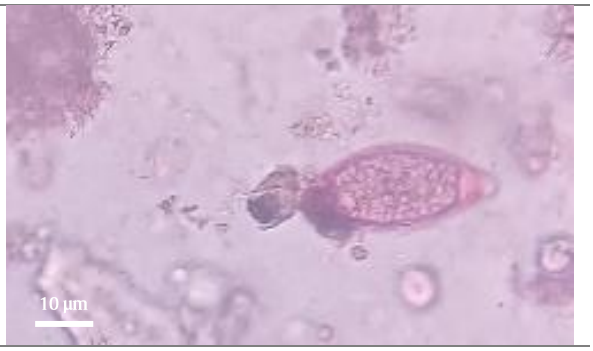
Table 2. Microscopic observation of infused assessment results for hibiscus and roselle flowers

	
The results of staining using 2% eosin dye on *Ascaris lumbricoides* eggs obtained a score of 3 (good) because the visual field was contrasting, the worm eggs absorbed the color and the parts of the worm eggs were clearly visible. Magnification 100x.	The results of staining using dye soaked in hibiscus flowers on *Ascaris lumbricoides* eggs obtained a score of 2 (not good) because the visual field lacked contrast, the worm eggs did not absorb the color and the parts of the worm eggs were clearly visible. Magnification 100x.
	
The results of staining using dye soaked in hibiscus flowers on *Ascaris lumbricoides* eggs obtained a score of 1 (not good) because the visual field did not have contrast, the worm eggs did not absorb the color and the parts of the worm eggs were not clearly visible. Magnification 100x.	The results of staining using dye soaked in rosella flowers on *Ascaris lumbricoides eggs* obtained a score of 2 (not good) because the visual field lacked contrast, the worm eggs did not absorb the color and the parts of the worm eggs were clearly visible. Magnification 100x.

The results of staining using dye soaked in rosella flowers on *Ascaris lumbricoides eggs* obtained a score of 1 (not good) because the visual field did not have contrast, the worm eggs did not absorb the color and the parts of the worm eggs were not clearly visible. Magnification 100x.

The results of staining using 2% eosin dye on *Trichuris trichiur a eggs* obtained a score of 3 (good) because the visual field contrasted, the worm eggs absorbed the color and the parts of the worm eggs were clearly visible. Magnification 100x.

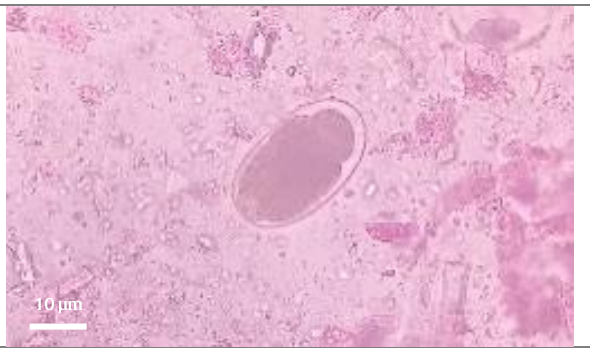
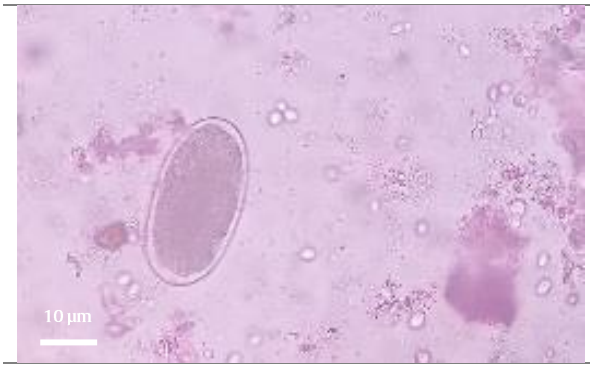
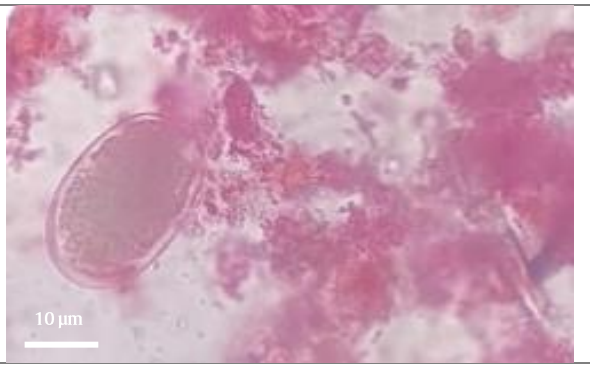
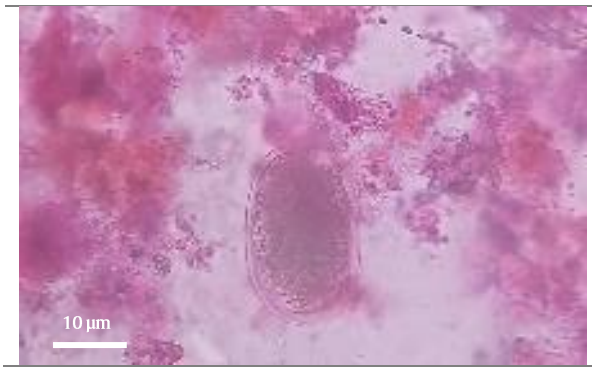
The results of staining using dye soaked in hibiscus flowers on *Trichuruis trichiura* eggs obtained a score of 2 (not good) because the visual field lacked contrast, the worm eggs did not absorb the color and the parts of the worm eggs were clearly visible. Magnification 100x.



The results of staining using dye soaked in hibiscus flowers on *Trichuris trichiura* eggs obtained a score of 1 (not good) because the visual field did not have contrast the worm eggs did not absorb the color and the parts of the worm eggs were not clearly visible. Magnification 100x.

The results of staining using dye soaked in rosella flowers on *Trichuruis trichiura* eggs obtained a score of 2 (not good) because the visual field lacked contrast, the worm eggs did not absorb the color and the parts of the worm eggs were clearly visible. Magnification 100x.

The results of staining using dye soaked in rosella flowers on *Trichuris trichiura eggs* obtained a score of 1 (not good) because the visual field did not have contrast, the worm eggs did not absorb the color and the parts of the worm eggs were not clearly visible. Magnification 100x.

	
The results of staining using 2% eosin dye on *hookworm eggs* obtained a value of 3 (good) because of the contrast field of view, the worm eggs absorb the color and the worm egg parts are clearly visible. Magnification 100x.	The results of staining using dye soaked in hibiscus flowers on *hookworm eggs* obtained a score of 2 (not good) because the visual field lacked contrast, the worm eggs did not absorb the color and the parts of the worm eggs were clearly visible. Magnification 100x.
	
The results of staining using dye soaked in hibiscus flowers on *hookworm eggs* obtained a score of 1 (not good) because the visual field did not have contrast, the worm eggs did not absorb the color and the parts of the worm eggs were not clearly visible. Magnification 100x.	The results of staining using dye soaked in rosella flowers on *hookworm eggs* obtained a score of 2 (not good) because the field of view lacked contrast, the worm eggs did not absorb the color and the parts of the worm eggs were clearly visible. Magnification 100x.
	The results of staining using dye soaked in rosella flowers on *hookworm eggs* obtained a score of 1 (not good) because the field of view did not have contrast. the worm eggs did not absorb the color and the parts of the worm eggs were not clearly visible. Magnification 100x.

3.2. Results Statistical Analysis of Infused Hibiscus And Rosella Flowers

The results of the statistical assessments were carried out by the Kruskal Wallis and Mann-Whitney U tests. The Kruskal Wallis test was carried out to find out whether there are differences between two or more variables, while the Mann-Whitney U test was carried out to find significant differences between two variables (16). The results of the Kruskal Wallis and Mann-Whitney U tests are said to have differences if the significant (p-value) is 0.05 (17). The results of the Kruskal Wallis and Mann-Whitney U tests are presented in the Table 3 and 4.

This research uses alternative dyes derived from plants, hibiscus and roselle flowers. The use of hibiscus and rosella flowers in this study is because these plants contain anthocyanins. Anthocyanins are compounds that have atmospheric properties which have the advantage of reacting with acids and bases. Anthocyanin compounds found in fruits, vegetables and tubers. The pH level can also affect the colour of anthocyanins. At acidic pH, anthocyanins will be red or purple, while turn green or yellow at alkaline pH (10). The anthocyanin content found in hibiscus flowers is anthocyanin which comes from the pelargonidin, peonidin, cyanidin, malvidin, petunidin and delfinidin groups (12). Moreover, the anthocyanin content found in rosella flowers is anthocyanin pigment. Anthocyanin functions as an antioxidant process that naturally occurs which is believed to be cure degenerative diseases. Additionally, Anthocyanin produces a red colour, which is used as a natural dye for making food ingredients and other alternative colourings (18). This study also used 2% eosin dye as a control. Eosin is a dye solution often used in microscopic examination of faeces to detect the presence of protozoa and worm eggs and is used as a stool diluent. Examination of worm parasite eggs using a 2% eosin reagent aims to differentiate between worm eggs and faeces (15).

Table 3. Kruskal Wallis test results of infused hibiscus and roselle flowers

Concentration	n	Mean Rank	Chi square	Df	Asymp. Sig.
Hibiscus flowers. *Ascaris* 1:1	4	58.00	44.159	24	0.007
Hibiscus flowers. *Ascaris* 1:2	4	46.00			
Hibiscus flowers. *Ascaris* 1:3	4	34.00			
Hibiscus flowers. Ascaris 1:4	4	34.00			
Hibiscus flowers.*Trichuris trichiura* 1:1	4	58.00			
Hibiscus flowers. *Trichuris trichiura* 1:2	4	58.00			
Hibiscus flowers. *Trichuris trichiura* 1:3	4	46.00			
Hibiscus flowers. *Trichuris trichiura* 1:14	4	58.00			
Hibiscus flowers. *Hokworm* 1:1	4	58.00			
Hibiscus flowers. *Hokworm* 1:2	4	46.00			
Hibiscus flowers. *Hokworm* 1:3	4	58.00			
Hibiscus flowers. *Hokworm* 1:4	4	34.00			
Roselle flowers. *Ascaris* 1:1	4	58.00			
Roselle flowers. *Ascaris* 1:2	4	46.00			
Roselle flowers. *Ascaris* 1:3	4	34.00			
Rosella flowers. *Ascaris* 1:4	4	34.00			
Roselle flowers. *Trichuris trichiura* 1:1	4	58.00			
Roselle flowers. *Trichuris trichiura* 1:2	4	58.00			
Roselle flowers. *Trichuris trichiura* 1:3	4	58.00			
Roselle flowers. *Trichuris trichiura* 1:4	4	58.00			
Roselle flowers *Hokworm* 1:1	4	46.00			
Roselle flowers. *Hookworm* 1:2	4	58.00			
Roselle flowers. *Hokworm* 1:3	4	34.00			
Roselle flowers. *Hokworm* 1:4	4	34.00			
Eosin 2% is control	4	98.50			
Total	100				

Based on the research, the results of varying concentrations of 1:1 and 1:2 in the hibiscus and rosella flower bath which can be effectively used as alternative dyes for examination of soil-transmitted helminth eggs due to the contrast field of view, worm eggs absorb colour, and egg parts worms are visible. This result follows the research of Bosch F, et al. (19) that examination of soil-transmitted helminths eggs using dye soaked in teak leaves (*Tectona grandis* Linn F) on *A. lumbricoides* eggs results in the albumin, glycogen, and lipoidal layers of the egg being reddish brown to dark brown in colour, while the morula is brownish yellow with a light background so that it can be easily it is easy to distinguish between worm eggs and faeces. In *T. trichiura* eggs, the colour results in the vitelline and chitin layers becoming dark brown and lipids brown, while the morula is light brown with a light

brown background so that it can easily distinguish between worm eggs and faeces. In hookworm eggs, the colour results in the apparent coloured egg wall layer. The vitelline becomes dark brown to reddish and the morula is grey with a light background so that it can easily distinguish between worm eggs and faeces. In this study, variations in concentrations of 1:3 and 1:4 in infusions of hibiscus and roselle flowers gave poor staining results due to observations using the same composition giving the field of vision was not in contrast, worm eggs absorb less colour and the parts of worm eggs are not seemingly visible. Variations in concentrations of 1:3 and 1:4 in hibiscus and roselle flower infusions did not give effective staining results due to differences in the colours produced. One of the variations in the concentration of the immersion is due to the difference in pH between eosin and each concentration variation in staining (20). Factors other than pH that can affect anthocyanin stability in plants are the process of glycolysis, temperature, light and the presence of other metal ions (21).

Table 4. Man-Whitney U test results of infused hibiscus and roselle flowers

Concentration	*Mean difference*	*Asymp.Sig*
Hibiscus flowers 1:1 – Hibiscus flowers 1:2	2.00	0.317
Hibiscus flowers 1:1 – Hibiscus flowers 1:3	1.83	0.127
Hibiscus flowers 1:1 – Hibiscus flowers 1:4	1.67	0.127
Hibiscus flowers 1:2 – Hibiscus flowers 1:3	2.00	0.495
Hibiscus flowers 1:2 – Hibiscus flowers 1:4	2.00	0.495
Hibiscus flowers 1:3 – Hibiscus flowers 1:4	2.00	1.000
Roselle flowers 1:1 – Roselle flowers 1:2	2.00	0.317
Roselle flowers 1:1 – Roselle flowers 1:3	2.00	0.127
Roselle flowers 1:1 – Roselle flowers 1:4	2.00	0.127
Roselle flowers 1:2 – Roselle flowers 1:3	2.00	0.495
Roselle flowers 1:2 – Roselle flowers 1:4	2.00	0.495
Roselle flowers 1:3 – Roselle flowers 1:4	1.92	1.000
Hibiscus flowers 1:1 – eosin 2%	1.92	0.008
Hibiscus flowers 1:2 – eosin 2%	1.92	0.011
Hibiscus flowers 1:3 – eosin 2%	1.92	0.013
Hibiscus flowers 1:4 – eosin 2%	1.92	0.013
Roselle flowers 1:1 – eosin 2%	1.92	0.008
Roselle flowers 1:2 – eosin 2%	1.92	0.011
Roselle flowers 1:3 – eosin 2%	1.92	0.013
Roselle flowers 1:4 – eosin 2%	1.92	0.013

Utilization of alternative dyes using infusions of hibiscus and rosella flowers showed quite good results when observed microscopically because the background colour was quite contrasting, the worm eggs did not absorb the colour and the egg part was seemingly visible. Apart from that, the colour of the eggs and faeces looks different and can be easily distinguished. According to the observations, worm egg preparations with alternative colouring infusions in hibiscus and roselle flowers do not make the eyes sore, hot and tired. Apart from that, in terms of cost, it is economical, can be found around the living environment and is also environmentally friendly. Infused hibiscus flowers and rosella flowers can be used as a natural stain for examining soil-transmitted helminths eggs, but the results are not as good as eosin (15). Indeed, the observed differences in color resulting from each variation in immersion concentration could be attributed to various factors, including the pH variance between eosin and the different concentration variations in coloring agents. pH levels play a crucial role in dye absorption and color development, influencing the overall staining outcomes. The distinct pH levels of eosin and the alternative staining agents may lead to varying degrees of color intensity and hue, contributing to the differences observed in the staining results (22). Factors other than pH that can influence the stability of anthocyanins in plants are the glycolysis process, temperature, light and the presence of other metal ions (23).

4. CONCLUSIONS

In the research comparing the efficacy of infused hibiscus (*Hibiscus rosa-sinensis* L.) and roselle flowers (*Hibiscus sabdariffa* L.) as alternative staining agents for examining soil-transmitted helminths (STH) eggs against 2% eosin as the control, the null hypothesis (H0) is rejected, while the alternative hypothesis (H1) is accepted. This signifies a notable difference in effectiveness between infused hibiscus and roselle flowers compared to 2% eosin

in preparing STH egg samples. Specifically, the infusion of hibiscus and roselle flowers at a 1:1 concentration ratio demonstrates promising potential as an alternative staining method for STH egg examination. Statistical analysis confirms a significant difference ($p < 0.005$) between the staining outcomes of infused hibiscus and roselle flowers compared to the conventional 2% eosin staining.

Author contributions: AMC: Methodology, formal analysis, resources, supervision, project administration, funding acquisition, writing-review and editing; EOR: Data curation, writing-original draft preparation; FA: Investigation, visualization; AMC, EOR: Con-ceptualization; AMC, EOR, FA: Software, validation. All authors have read and agreed to the published version of the manuscript.

Funding: None.

Acknowladgements: The author would like to Anwar Medika University, express gratitude to all parties whohave participated and assisted in the implementation of the research, as well as the respondent who have been involved in this study.

Ethics statement: None. Studies involving humans and animals must have been performed with the approval of an appropriate ethics committee and provide the reference number.

Conflict of interest: There are no confilict of interest.

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