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ANTHOCYANIN-CONTAINING PLANT EXTRACTS AS AN ALTERNATIVE DYE FOR MICROSCOPIC EXAMINATION OF SOIL-TRANSMITTED HELMINTHS

ANTOSIANIN PADA EKSTRAK TANAMAN SEBAGAI PEWARNA ALTERNATIF UNTUK PENGAMATAN MIKROSKOPIS SOIL TRANSMITTED HELMINTHS

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ABSTRACT

Background: *Eosin is commonly used for microscopic examination of Soil-Transmitted Helminth (STH) infections, but natural anthocyanin-based pigments remain underutilized.* ***Purpose:*** *This study evaluates the potential of anthocyanins extracted from red beans (Phaseolus vulgaris), hibiscus flowers (Hibiscus rosa-sinensis), and amaranth leaves (Amaranthus tricolor) as eosin alternatives for staining STH eggs in stool smears.* ***Method:*** *Extracts were obtained using 96% ethanol for 24, 48, and 72 hours, with nine replications. Stool preparations were stained with these extracts and compared to eosin, assessing color intensity, contrast, and egg layer clarity.* ***Result:*** *Red bean extracts scored 2.1, 2.7, and 2.8 at 24, 48, and 72 hours, with the latter two showing no significant difference from eosin. Hibiscus flower extracts scored 1.6, 2.2, and 2.8, with the 72 hours extract comparable to eosin. Amaranth leaf extracts scored 1.4, 1.7, and 1.9, all significantly different from eosin.* ***Conclusion:*** *Red bean extracts (48 and 72 hours) and hibiscus flower extract (72 hours) provided staining comparable to eosin, with red bean extract being the most promising alternative. These findings suggest that anthocyanin-based stains can serve as viable substitutes for eosin in diagnosing helminthiasis via stool smear microscopy.*

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ABSTRAK

Latar belakang: Eosin umumnya digunakan untuk pemeriksaan mikroskopis infeksi *Soil-Transmitted Helminth* (STH), tetapi pigmen berbasis antosianin alami masih belum dimanfaatkan secara maksimal. **Tujuan:** Penelitian ini mengevaluasi potensi antosianin yang diekstrak dari kacang merah (*Phaseolus vulgaris*), bunga kembang sepatu (*Hibiscus rosa-sinensis*), dan daun bayam (*Amaranthus tricolor*) sebagai alternatif eosin untuk pewarnaan telur STH pada apusan tinja. **Metode:** Ekstrak diperoleh menggunakan etanol 96% selama 24, 48, dan 72 jam, dengan sembilan kali ulangan. Sediaan tinja diwarnai dengan ekstrak ini dan dibandingkan dengan eosin, untuk menilai intensitas warna, kontras, dan kejernihan lapisan telur. **Hasil:** Ekstrak kacang merah memperoleh skor 2,1; 2,7; dan 2,8 pada 24, 48, dan 72 jam, dengan dua yang terakhir tidak menunjukkan perbedaan signifikan dari eosin. Ekstrak bunga kembang sepatu memperoleh skor 1,6; 2,2; dan 2,8, dengan ekstrak 72 jam sebanding dengan eosin. Ekstrak daun bayam memperoleh skor 1,4; 1,7; dan 1,9, semuanya berbeda secara signifikan dari eosin. **Kesimpulan:** Ekstrak kacang merah (48 dan 72 jam) dan ekstrak bunga kembang sepatu (72 jam) memberikan pewarnaan yang sebanding dengan eosin, dengan ekstrak kacang merah menjadi alternatif yang paling menjanjikan. Temuan ini menunjukkan bahwa pewarna berbasis antosianin dapat berfungsi sebagai pengganti eosin yang layak dalam mendiagnosis helminthiasis melalui mikroskopi apusan tinja.

Kata kunci:
Antosianin pada ekstrak tanaman, Ekstraksi etanol, Pewarna alternatif, STH

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INTRODUCTION

Clinical laboratories in the modern world of health significantly contribute to diagnosing and managing diseases, especially because of their role in measuring biomarkers and identifying species of microorganisms in specimens or body tissues. This role covers a wide range of disciplines, including clinical chemistry, hematology, clinical microbiology, clinical immunology, and molecular diagnostics, supported by a combination of advanced technology, skilled professionals, and effective management.

In the scope of clinical microbiology, microscopic examination serves as a gold standard for diagnosing diseases caused by parasites, especially helminths, protozoa, and arthropods, with the most representative specimens being blood and feces (Rifai *et al*., 2022). This microscopic examination is usually performed on a slide stained with certain dyes to detect microorganisms such as helminth eggs. Typical dyes like iodine, safranin, trypan blue, and eosin Y are used to identify infections caused by *Soil-Transmitted Helminths* (STH). When compared to the culture approach, the microscopic method is considered more time-effective (Amoah *et al*., 2017).

Synthetic dyes' disadvantages include high cost, limited availability, inability to produce independently, potential for irritation, and the need for potentially dangerous solvents. Therefore, reliance on them should be avoided. In addition, synthetic dyes are reported to be toxic to helminth embryos, therefore they can be detrimental if the examination requires observation of live microorganisms (Amoah *et al*., 2017) for this reason, alternative materials that are safe and can address the aforementioned problems are needed.

Anthocyanin is a well-known natural dye that can be found around us. This pigment is a compound from the flavonoid group, which gives plants a red, blue, or purple color, and has an antioxidant effect (Khoo *et al*., 2017). Anthocyanins can be found in red beans (*Phaseolus vulgaris*) (Li *et al*., 2021; Liu *et al*., 2023; Meenu *et al*., 2023), hibiscus (*Hibiscus rosa-sinensis*) (Kalpana *et al*., 2021; Pieracci *et al*., 2021), and amaranth (*Amaranthus tricolor*) (Sarker *et al*., 2022; Sarker and Oba, 2020).

Plants have been extensively studied as sources of bioactive substances such as anthocyanins, however less is known about their potential as alternative substances for laboratory diagnoses, particularly in staining parasitological smears. This research aims at exploring the feasibility of using anthocyanins extracted from plants such as red beans, hibiscus flowers, and amaranth leaves as substitutes for eosin in staining parasitological smears to identify STH eggs.

MATERIAL AND METHOD

Preparation of anthocyanin-containing plant extract

The natural plants used were obtained from different locations: red beans and amaranth were purchased from a traditional market, while hibiscus flowers were picked from a nearby garden. The selection of materials was based on their physical state, which included being undamaged, free of decay, and free of discoloration to minimize the possibility of natural substances being lost or destroyed. All of these materials were then washed to remove dirt and unnecessary materials. The extraction procedure used the maceration method based on Sugiharto *et al*. (2020) with slight modifications. One hundred grams of each ingredient were soaked in 100 mL of 96% ethanol in three erlenmeyer flasks, sealed with aluminum foil, and stored away from direct sunlight. Maceration of each ingredient was conducted for 24, 48, and 72 hours. There were 9 replications for each ingredient and each maceration time. Filtration was performed using Whatman filter paper after each maceration time for each ingredient.

Qualitative tests on anthocyanins were carried out by administering 2M NaOH drop by drop into a test tube. The red color changed to blue-green and disappeared slowly, proving the presence of anthocyanins (Anggriani *et al*., 2017),

Stool samples

The feces used in this research were obtained from the Laboratory of Parasitology, Pontianak Ministry of Health Polytechnic, and have been confirmed to contain STH eggs. Prior to use, feces were stored at 4 °C, not exposed to direct sunlight, and not used for other examinations.

Staining of stool smears using anthocyanin-containing plant extracts

A stool sample was collected using the tip of the stick to prepare a direct smear. The stool sample was flattened to form a circle with a diameter of 1 - 2 cm, followed by the application of 1 - 2 drops of natural extract onto the smear. The smear was then covered with a cover glass and examined under the light microscope at magnifications of 100 and 400 times. The scoring method for staining quality was based on (Oktari and Mutamir, 2017) and is shown in Table 1.

Examination of the quality of staining

The quality of anthocyanin staining from three extracts was evaluated using microscopy examination of STH-positive stool samples. The examinations included assessing distinctness and contrast, the effective

absorbance of staining by helminth eggs, and the discernibility of the parts of the egg, all of which were scored based on visual perception, to minimize bias in scoring, visual observations were conducted by a single person.

Statistical analysis

The size of the replication unit is determined based on the number of treatment groups (24, 48, and 72 hours) of each natural plant and is calculated using the Federer formula. Based on the calculation, 9 replication units are needed for each treatment group per natural plant, so there are 27 replications in each natural plant and 78 in total. The *Mann-Whitney* test was used to compare the average scores between each plant extract of each maceration time and the control (2% eosin). The *Mann-Whitney U* test is a non-parametric test used to determine the difference in medians of two independent groups when the data scale for the dependent variable is ordinal or interval/ratio but is not normally distributed. The statistical software used in this study was JASP.

RESULT

Anthocyanin-containing plant extracts

Qualitative tests with 2M NaOH showed that all extracts obtained contained anthocyanins. Based on the duration of extraction time, it was observed that the longer the extraction period, the redder the color of the red bean extract, the thinner the consistency, the less foam, and the clearer the color (Figure 1).

Based on the extraction time, the characteristics of the hibiscus flower extract include a red color with no color gradation, a thin consistency (thinner than red bean and amaranth leaf extracts), absence of foam, and clarity in all treatments (Figure 2).

The characteristics of the amaranth leaf extract obtained by extraction from 24 to 72 hours include a red color that gradually changes to a purple, a thicker consistency than two other plant extracts, persistent foam in all extraction treatment, and high turbidity at the beginning of extraction that decreases as the extraction period is extended to 72 hours (Figure 3).

Scoring of the quality of staining

Microscopic examinations of STH-positive stool samples stained by three different extracts revealed clear distinctness and contrast, allowing for the morphology of helminth eggs to be recognized in detail. Anthocyanin was also effectively absorbed by the eggs, and smears stained with extracts derived from a longer extraction time provided a more contrasting picture. Table 2 shows the staining quality scores of STH-positive stool samples stained with each of the three extracts, while Figures 4 - 6 show the staining quality characteristics at 100 times magnification.

Statistical analysis

The results of statistical tests using JASP software showed that microscopic examination using 24 hours red bean extract obtained a significantly different score from the control (*p-value* 0.002 <0.05), while the 48 and 72 hours extracts were not significantly different, with *p-values* of 0.298 and 0.585, respectively. Furthermore, microscopic examination using 24 and 48 hours hibiscus flower extract showed a significant difference in score from the control, with *p-values* < 0.001 and 0.007 (<0.05), respectively, while the 72 hours extract was not significantly different from the control, with a *p-value* of 0.585. Finally, the microscopic observation scores of 24, 48, and 72 hours amaranth leaf extracts obtained *p-values* < 0.001 (<0.05) respectively, which means that all three showed significant differences from the control. Table 3 shows the results of statistical analysis using the *Mann-Whitney* test to evaluate the differences between each treatment group compared with the control.

Table 1. Scoring of staining quality

Score	Microscopic observation
1	Helminth eggs do not absorb color and the field lacks contrast, making it difficult to see some portions of the egg
2	Helminth eggs absorb less color, the field lacks contrast, and portions of the egg are less clearly apparent
3	Helminth eggs absorb color effectively and are easily apparent in portions due to the contrasting appearance of the visual field

Table 2. Scores for the quality of staining of *Soil-Transmitted Helminths* (STH) positive stool samples stained with extracts of red beans, hibiscus flowers, and amaranth leaves

Samples	Treatments	Minimum	Maximum	Median	Mean
Red bean extract	24 hours	2	3	3	2.1
	48 hours	2	3	3	2.7
	72 hours	2	3	3	2.8
Hibiscus flower extract	24 hours	1	2	2	1.6
	48 hours	2	3	2	2.2
	72 hours	2	3	3	2.8
Amaranth leaf extract	24 hours	1	2	1	1.4
	48 hours	1	2	2	1.7
	72 hours	1	2	2	1.9
Control	Eosin 2%	2	3	3	2.9

Table 3. Comparison of staining quality scores between control and maceration time of each anthocyanin containing plant extract by *Mann-Whitney* analysis

Samples	Treatments (hours)	Control	*p-value*
Red bean extract	24	Eosin 2%	0.002
	48 *		0.298
	72 *		0.585
Hibiscus flower extract	24		< 0.001
	48		0.007
	72 *		0.585
Amaranth leaf extract	24		< 0.001
	48		< 0.001
	72		< 0.001

*Not significantly different (*p-value* ≥ 0.05)

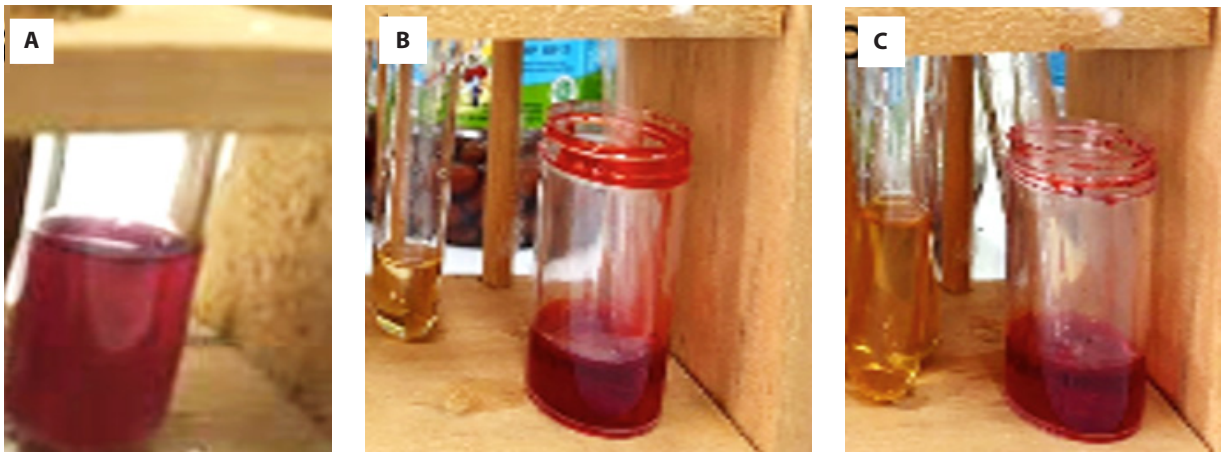

Figure 1. Red bean extract (A) 24 hours, (B) 48 hours, and (C) 72 hours

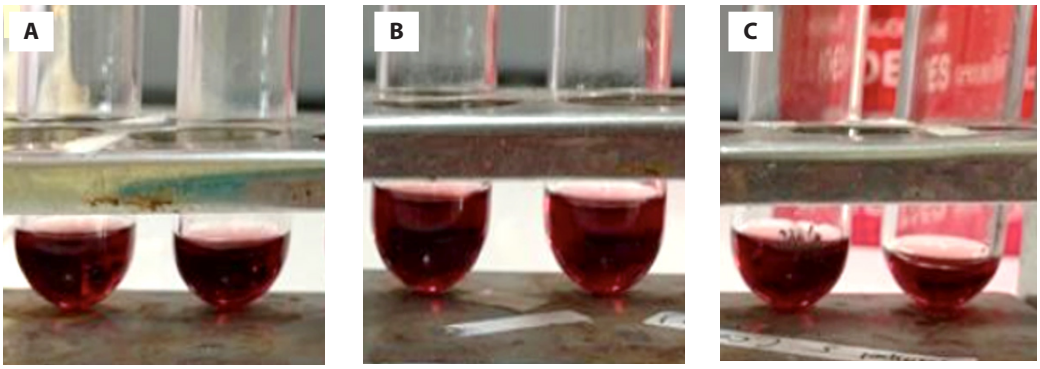

Figure 2. Hibiscus flower extract (A) 24 hours, (B) 48 hours, and (C) 72 hours

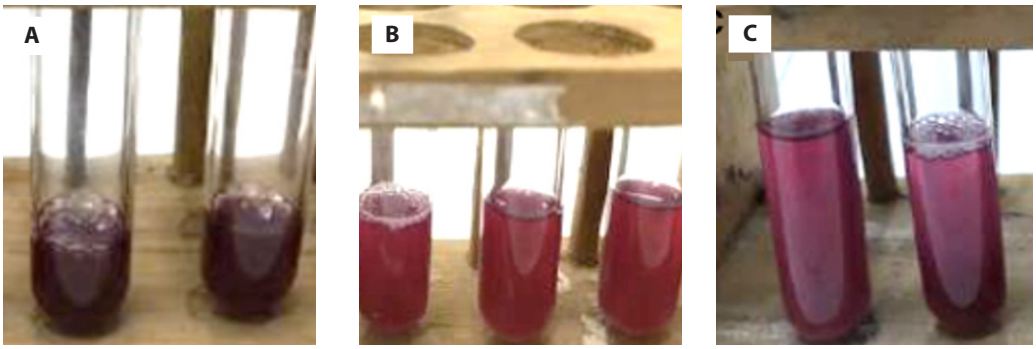

Figure 3. Amaranth leaf extract (A) 24 hours, (B) 48 hours, and (C) 72 hours

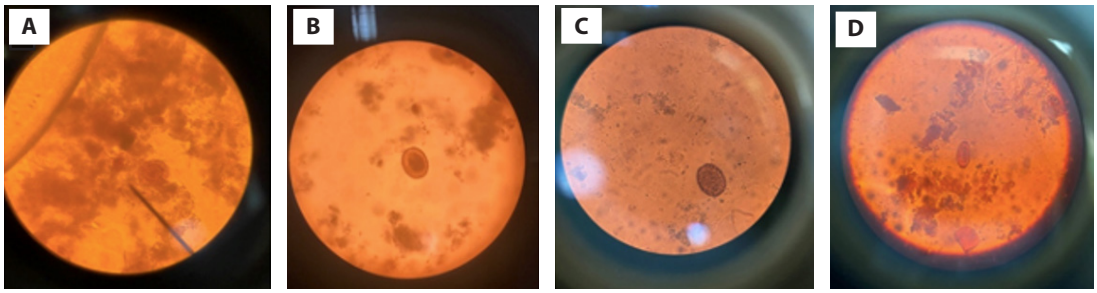

Figure 4. Microscopic examination of red bean extract staining for (A) 24 hours, (B) 48 hours, (C) 72 hours, and (D) control

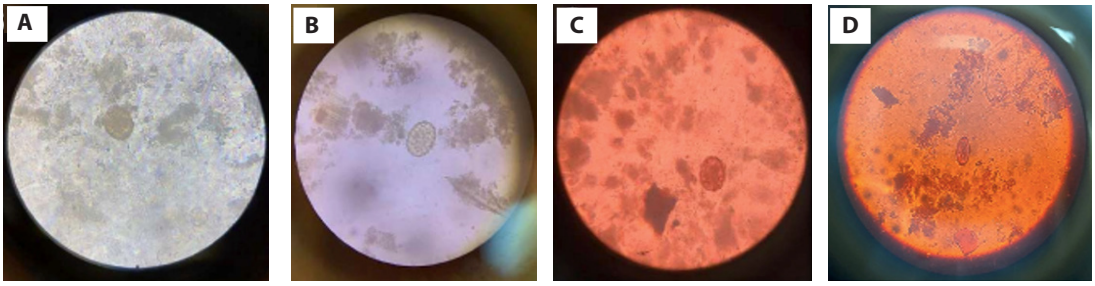

Figure 5. Microscopic examination of hibiscus flower extract staining for (A) 24 hours, (B) 48 hours, (C) 72 hours, and (D) control

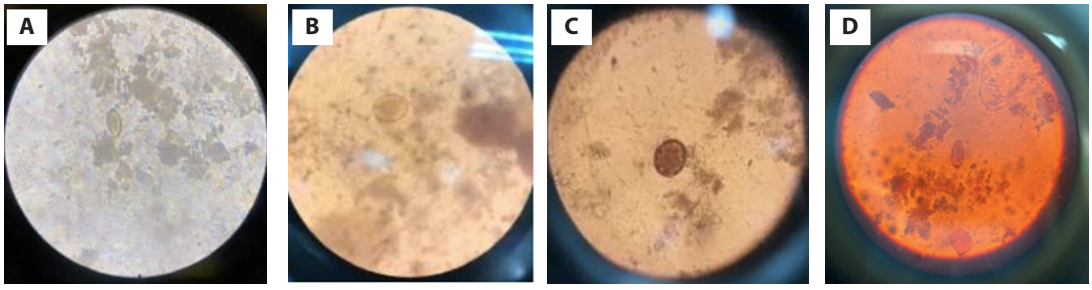

Figure 6. Microscopic examination of amaranth leaf extract staining for (A) 24 hours, (B) 48 hours, (C) 72 hours, and (D) control

DISCUSSION

Factors that influence whether a natural dye can be used in staining include availability, suitability, color stability, and non-toxicity (Sachdev *et al*., 2021). Anthocyanin-containing plant extracts used as alternatives to eosin have several advantages such as being easy to obtain, not posing danger to workers, and not causing damage or interference with examination results. As explained earlier, these three plant extracts contain anthocyanins, one of the abundant natural pigments (Oliveira Filho *et al*., 2021), and have the potential to be utilized as dyes for staining stool smears prior to identifying STH eggs. Not all natural dyes can serve as an alternative dye, because the color they produce is different, while smears stained with anthocyanin show results similar to those stained with 2% eosin.

Anthocyanins, a type of flavonoid, are effective for adding color to plants or their by products when combined with other pigments. This substance also possesses pharmacological qualities, such as activities that reduce capillary fragility and permeability, as well as antioxidant, anti-inflammatory, and anti-edema capabilities (Lozada-Ramírez *et al*., 2021). These pigments are natural dyes that give many foliage, flowers, vegetables, and fruits-especially berries-their stunning red-orange to blue-purple hues. Since the beginning of the twenty-first century, research on anthocyanins has focused on their potential health-improving effects and their use as an alternative to synthetic food coloring (Alappat and Alappat, 2020; Backes *et al*., 2020; Lu *et al*., 2021; Wallace and Giusti, 2019). This research is based on their benefits, which include being natural, non-toxic, and water-soluble (Gecchele *et al*., 2021). Recently, with the same benefits, research on the use of anthocyanin-containing natural substances has been conducted, particularly in the diagnosis of STH infections. According to preliminary findings, the longer the extraction time, the more intense and clearer the color of the suspension, indicating a higher anthocyanin content. These traits were also observed in the staining and microscopic examination of stool smears containing STH.

The amount of anthocyanin in plant extracts varies greatly. Aquino-Bolaños *et al*. (2016) measured the dry weight of 26 different types of red beans and found anthocyanin levels ranging from 0.04 to 9.07 mg/g. Meanwhile, Rodríguez Madrera *et al*. (2021) measured the monomeric anthocyanin content and found results ranging from 120 to 1623 mg/g. The total anthocyanin content of *H. rosa-sinensis* has been estimated to be around 359.3 mg cyanidin 3-glucoside/100 g dry weight. The same study discovered four different anthocyanins in acidified hibiscus ethanol extract, with delphinidin-3-O-sambubioside and cyanidin-3-O-sambubioside having the highest concentrations (19.9 mg/g dry weight and 16.13 mg/g dry weight, respectively) (Amer *et al*., 2022). The amount of anthocyanin found in this plant, 0.59 g/mL when extracted with distilled water, can alter with time and increasing PH (Madushan *et al*., 2021). Anthocyanin concentrations were found to range from 690 to 2285 mg/100 g dry weight in four different species of amaranth plants (Yang *et al*., 2020). Anthocyanin levels reached 16.23 and 15.65 mg/g dry weight in research on red spinach seedlings maintained at temperatures of 5 and 10 °C for 3 days; these levels rose further if storage was extended to 7 days (Wittayathanarattana *et al*., 2022). In this research, although we did not identify other compounds or quantify anthocyanin levels, the qualitative tests we conducted with 2M NaOH confirmed the presence of these natural pigments in all extracts.

According to the study's findings, red beans extracted for 48 and 72 hours yield an anthocyanin-containing solution that can produce microscopic examination results identical to those of eosin, particularly when it comes to seeing STH eggs. The similar outcome was reached from the extract of hibiscus flowers extracted for 72 hours. However, extraction up to 72 hours of amaranth leaf extract still obtained a solution of poor quality compared to the control for microscopic examination. This indicates that red bean extract is thought to be the best option among the others, and the longer the natural material is extracted, the better the solution will be for microscopic analysis. Therefore, anthocyanin-containing substances extracted by this method can be used as alternative dyes to stain and diagnose helminthiasis by microscopic examination of STH stool smears.

However, the color of anthocyanin-containing plant extracts may change over time, and their efficacy decreases with storage duration. According to Alappat and Alappat (2020), anthocyanin color is susceptible to pH, light, temperature, and metal ions, and the enzyme polyphenol oxidase has been identified as one of the causes of anthocyanin color destabilization (Hossain *et al*., 2016). In addition, the stability of anthocyanins is also influenced by various factors, including inter- and intramolecular complexes. At the molecular level, the degree of hydroxylation/methoxylation of the anthocyanidin B ring and the nature of the sugar and/or acid conjugation have the greatest influence on the color produced by these pigments. An increase in the number of hydroxyl and/or methoxyl groups in the B ring of anthocyanidin results in a shift in the maximum visible absorption wavelength, giving rise to a bluish effect on the resulting color. Substitution of the R group on ring B can also affect pigment stability, where hydroxylation of ring B has been shown to reduce the stability of anthocyanins, while methoxylation produces the opposite effect. Acylation of sugar substitutions and/or anthocyanidins can also produce bathochromic and/or hyperchromic

shifts (increased absorption), thereby changing the spectrum of a compound (Wallace and Giusti, 2019). Therefore, anthocyanin-containing plant extracts in this current research should be used within 12 hours after extraction to avoid color changes and further misdiagnosis of STH infection.

The anthocyanin-containing substances extracted using the method above are not long-lasting because the color can change within at least 12 hours after extraction, suggesting that the substances should be used immediately after extraction. Therefore, further research is required to incorporate preservatives into this substance so that it can be stored and used for a prolonged time.

CONCLUSION

The microscopic analysis of stool smears, the study's findings demonstrated that red beans extracted for 48 and 72 hours and hibiscus flowers extracted for 72 hours produced solutions that were comparable to 2% eosin, particularly for identifying STH. Therefore, natural substances can be considered as an alternative to eosin for staining stool smears, especially for diagnosing STH infections. Natural components have additional value because they are typically nontoxic, easy to obtain, and inexpensive, although not long-lasting.

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