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Modification of the Kato-Katz Diagnosis Technique Using "Canang" Flower Waste Extract as a Staining for *Ascaris lumbricoides* Eggs

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ABSTRACT

Helminthiasis, particularly that caused by *Ascaris lumbricoides*, is a major global health issue, especially in areas with poor sanitation. One method for preventing and controlling *Ascaris lumbricoides* infection is through identification via the Kato-Katz diagnostic technique. The use of synthetic dyes, such as methylene blue, in the Kato-Katz method raises concerns for both human health and the environment. In humans, methylene blue causes skin irritation, gastrointestinal issues upon ingestion, and systemic effects. Furthermore, its environmental impact includes reducing light penetration and acting as a toxic component in food chains. An alternative approach involves utilizing post-use offerings from Hindu rituals in Bali, known as *canang*, which consist of flower components such as *Impatiens balsamina* L. and *Tagetes erecta*. The natural dyes found in these flowers serve as an alternative to traditional staining methods. This study examined the efficacy of flower extracts as stainings using the cellophane absorption test, helminth egg detection and morphology identification, pH test, and measurement of heavy metal concentration. The dye made from *canang* flower waste at 3% did not differ much from manufactured stainings. Thus, *canang* flower waste is a safe alternative.

1. Introduction

The Kato-Katz technique is globally acknowledged as the gold standard for diagnosing soil-transmitted helminthiasis, particularly *Ascaris lumbricoides*, owing to its high sensitivity and specificity in moderate to heavy infections (Mbong Ngwese *et al*. 2020; Bosch *et al*. 2021). This method employs synthetic dyes such as methylene blue and malachipreferredn, which are favored for their rapid staining, vivid coloration, and ease of use in laboratory settings (Tuti *et al*. 2023). Methylene blue, a synthetic thiazine dye, binds to parasite cytoplasm and host DNA to enhance microscopic visualization of helminth eggs (Bascica 2020). However, its application raises significant environmental and health concerns due to its toxicity, carcinogenicity, and persistence in ecosystems (Riwayati *et al*. 2019). Prolonged exposure can induce skin irritation, gastrointestinal distress, and

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hematological abnormalities, while environmental contamination disrupts aquatic ecosystems by reducing sunlight penetration and introducing hazardous chlorides and metals (Saputra 2022; Bistas & Sanghavi 2023). Malachite green, another synthetic alternative, shares similar drawbacks, including carcinogenicity and ecological toxicity (Oladoye *et al*. 2022). Consequently, this study investigates the viability of substituting methylene blue with a natural dye, in a modified Kato-Katz protocol.

Canang flower waste, comprising *Impatiens balsamina* and *Tagetes erecta* from Balinese Hindu rituals, offers a sustainable alternative. Daily production ranges from 0.8 kg (weekdays) to 2.4 kg (ceremonial days), presenting a scalable resource (Wijaya & Putra 2021). However, heavy metal contamination (e.g., lead in *Impatiens balsamina* and cadmium in *Tagetes erecta*) necessitates rigorous safety assessments (Liu *et al*. 2021). While plant-based dyes, such as red spinach extract, have demonstrated staining efficacy, the use of *canang* waste in helminth diagnostics remains unexplored, highlighting the novelty of this study (Artanti *et al*. 2024).

This research aims to 1) analyze the impact of varying concentrations of *canang* flower waste extract on the results of the cellophane absorption test in the modified Kato-Katz technique; 2) analyze the impact of varying concentrations of *canang* flower waste extract on the results of helminth egg detection and identification through the modified Kato-Katz technique; and 3) obtain an overview of the results of the pH test and analysis of heavy metal contamination when using *canang* flower waste extract in the modified Kato-Katz technique.

2. Materials and Methods

2.1. *Canang* Samples

Canang flower waste samples were collected in May 2024 from eight sites across Buleleng Regency, Bali, Indonesia, primarily at Hindu temple locations near Singaraja (Table 1). The collected samples consisted of *Tagetes erecta*, *Impatiens balsamina* (red), *Impatiens balsamina* (purple), and a combination of these three flower types. Following the collection, the flowers were sorted by species to facilitate subsequent analyses.

Table 1. *Canang* sampling locations

Sample location	Coordinate
Jagatnatha Singaraja Temple – Ganesha Shrine	8°06'43"S 115°05'27"E
Melanting Temple – Pabersihan Shrine	8°09'35"S 114°40'51"E
Melanting Temple – Ratu Niang Sakti Temple	8°09'34"S 114°40'50"E
Melanting Temple – Pasar Agung Temple	8°09'34"S 114°40'50"E
Melanting Temple – Penataran Agung Temple	8°09'36"S 114°40'50"E
Pulaki Temple – Pabersihan and Penyawangan Shrines	8°08'42"S 114°40'49"E
Pulaki Temple – Penataran Agung Pulaki Temple	8°08'44"S 114°40'49"E
Pabean Temple	8°08'40"S 114°40'52"E

2.2. Stored Biological Material

Helminth feces samples in this research were sourced from anonymized, unlinked stored biological material at the Parasitology Laboratory, Faculty of Medicine, Universitas Pendidikan Ganesha. The use of stored, anonymized samples is consistent with established protocols for parasitological research, ensuring both the integrity of diagnostic procedures and the protection of participant confidentiality. This approach enables the reliable detection and analysis of helminth eggs without requiring direct linkage to individual identities.

2.3. Materials

The research employed various materials and methods to analyze *canang* flower waste. Extraction was performed using 96% ethanol (Merck Millipore, Germany, Batch: K46141383). For cellophane preparation and absorption tests, glycerol, distilled water (produced with a Bio-Base Water Purification System), *canang* flower waste extract, and methylene blue (Merck KGaA, Germany, Batch: K50122170912) were utilized. Microscopic examination was conducted for the detection and morphological identification of helminth eggs. pH analysis and heavy metal testing involved pH paper, cellophane preparation solution, concentrated nitric acid (HNO_3) and sulfuric acid (H_2SO_4) (Merck KGaA, Germany, Batch: K46084931), 0.5N HNO_3, and ion-free water. Precise measurements were obtained using an atomic absorption spectrophotometer (AAS) and microscopes.

2.4. Sample Materials

Operational variables include control variables such as the type of cellophane, Kato-Katz kit, sample container, storage conditions, preservative, and Ascaris lumbricoides, infected feces samples. The independent variable is the concentration of *canang* flower waste extract. The dependent variables comprise cellophane absorption, helminth egg detection and morphological identification, pH measurement, and analysis of heavy metal contamination.

2.5. Flowers of *Canang* Wastes Extraction

The flowers were sorted into four groups: Tagetes erecta, *Impatiens balsamina* (purple), *Impatiens balsamina* (red), and a mixture of these types. Each group was cut into 500 gram portions, soaked in two liters of 96% ethanol for five days with daily stirring, then filtered and concentrated using a rotary evaporator at 70°C to obtain dense extracts (Octora & Waruwu 2022). Lutein and anthocyanin contents were analyzed via UV-Vis spectrophotometry at 360-550 nm and 465-560 nm, respectively. For helminth egg examination, cellophane was dissolved in methylene blue dye solutions (1-3%) mixed with glycerol and distilled water, then soaked for 24 hours in a covered container (Calvopina 2018) (Table 2).

2.6. Organoleptic Study of the Cellophane Absorption Test

The cellophane absorption test employed organoleptic assessment through questionnaires to evaluate color quality from *canang* flower waste extracts at varying concentrations of *Tagetes erecta* and *Impatiens balsamina*. Color quality in cellophane preparations and microscopic results were also assessed. Absorption was rated on a scale of 1 (low) to 4 (very good), with a control group for comparison. Six experts from the Biomedical Laboratory, Faculty of Medicine, Universitas Pendidikan Ganesha, conducted the evaluations.

2.7. The Detection and Identification of Helminth Egg Morphology Test

The detection and morphological identification of helminth eggs were conducted using preserved biological samples from the Parasitology Laboratory at the Faculty of Medicine, Universitas Pendidikan Ganesha. The procedure followed the Kato-Katz protocol as outlined by the WHO Bench Aids (2019), which involves preparing thick smears from stool samples, identifying and counting helminth eggs under a microscope, and recording results by sample code, parasite species, and quantity. Egg intensity was calculated by multiplying the number of eggs observed by 24, since each smear uses 41.7 mg of stool and 24 smears approximate one gram, yielding eggs per gram (EPG). The assessment focused on the ability of modified cellophane to reveal helminth eggs, with morphology categorized as (1) no findings, (2) eggs with abnormal morphology, or (3) eggs with normal morphology. All results were verified by specialist parasitology laboratory staff to ensure accuracy and reliability.

Table 2. Treatment variation of *Canang* flower waste extract concentration

Treatment	T1	T2	T3	C (-)	C (+)
Concentration (%)	1%	2%	3%	Methylene blue with helminth negative sample	Methylene blue with helminth positive sample

Description:

$$\text{Concrentration (\%)} = \frac{\textit{Extract volume}}{\textit{Solvent volume}} \text{ x } 100\%$$

2.8. pH Analysis and Heavy Metal Contamination Level Test

The pH test was conducted using pH paper, a standard method for assessing acidity or alkalinity by comparing color changes to a reference chart. After measurement, the cellophane preparation liquid was collected in a glass bottle sealed with aluminum foil and analyzed for heavy metal contamination at the Laboratory of the Faculty of Mathematics and Natural Sciences Laboratory, Universitas Pendidikan Ganesha. Sample preparation and atomic absorption spectrophotometer (AAS) analysis followed established protocols for accurate heavy metal quantification (Mantra & Widnyana 2022).

2.9. Data Analysis

The cellophane absorption test data are ordinal and thus analyzed using the Mann-Whitney test. Detection test data are nominal and evaluated with the Chi-Square Fisher's exact test, using the positive control for comparison. Egg counts per gram were further analyzed with a paired T-test against the positive control. Morphology test data are descriptive, focusing on helminth egg observations after staining with prepared cellophane. The analytical test data for the heavy metal contamination levels, measured as ratio data in ppm, were compared to Indonesian wastewater quality standards (Minister of Environment Regulation No. 5

of 2014, Appendix XLIV, point b). The significance level was set at a two-sided alpha of 0.05.

2.10. Ethical Clearance

The research has received ethical approval from the Ethics Committee of the Faculty of Medicine, Universitas Pendidikan Ganesha, with the ethical clearance number 032/UN48.24.11/LT/2024.

3. Results

3.1. Result of the Cellophane Absorption Test

Findings from Table 3 and Table 4 indicate significant differences between the various extract concentrations and the gold standard, suggesting comparable staining performance in the preparation solution and on the cellophane slide. However, microscopic color evaluation (Table 5) further showed no significant difference between the 3% extract group (T3) and the gold standard ($P > 0.05$). In comparison, the 1% (T1) and 2% (T2) groups differed significantly ($P < 0.01$), confirming 3% as the optimal concentration for staining.

3.2. Results of the Detection Test

All flower types, including Tagetes erecta, Impatiens balsamina (in purple and red), and their combination, achieved 100% positive detection (5/5 samples) with no negatives. Fisher's exact test confirmed the high effectiveness of all plant-based dyes in detecting *Ascaris lumbricoides* eggs, demonstrating significant diagnostic capability (Table 6).

Table 3. Results of the organoleptic test on the staining produced from the preparation solution procedure

Sample	Staining of cellophane preparation solution				Mann whitney test
	1	2	3	4	
Control	0 (0%)	0 (0%)	0 (0%)	6 (100%)	N/A
Tagetes erecta 1%	6 (100%)	0 (0%)	0 (0%)	0 (0%)	<0.01
Impatiens balsamina (purple) 1%	5 (83.3%)	1 (16.7%)	0 (0%)	0 (0%)	<0.01
Impatiens balsamina (red) 1%	4 (66.7%)	2 (33.3%)	0 (0%)	0 (0%)	<0.01
Mixed Flowers 1%	3 (50%)	3 (50%)	0 (0%)	0 (0%)	<0.01
Tagetes erecta 2%	1 (16.7%)	4 (66.7%)	1 (16.7%)	0 (0%)	<0.01
Impatiens balsamina (purple) 2%	1 (16.7%)	4 (66.7%)	1 (16.7%)	0 (0%)	<0.01
Impatiens balsamina (red) 2%	0 (0%)	4 (66.7%)	2 (33.3%)	0 (0%)	<0.01
Mixed Flowers 3%	0 (0%)	2 (33.3%)	4 (66.7%)	0 (0%)	<0.01
Tagetes erecta 3%	0 (0%)	1 (16.7%)	2 (33.3%)	3 (50%)	<0.01
Impatiens balsamina (purple) 3%	0 (0%)	1 (16.7%)	3 (50%)	2 (33.3%)	<0.01
Impatiens balsamina (red) 3%	0 (0%)	1 (16.7%)	3 (50%)	2 (33.3%)	<0.01
Mixed Flowers 3%	0 (0%)	2 (33.3%)	3 (50%)	1 (16.7%)	<0.01

Table 4. Results of the organoleptic test on the staining produced from the cellophane clide

Sample	Staining of cellophane slide				Mann whitney test
	1	2	3	4	
Control	0 (0%)	0 (0%)	0 (0%)	6 (100%)	N/A
Tagetes erecta 1%	6 (100%)	0 (0%)	0 (0%)	0 (0%)	<0.01
Impatiens balsamina (purple) 1%	5 (83.3%)	1 (16.7%)	0 (0%)	0 (0%)	<0.01
Impatiens balsamina (red) 1%	5 (83.3%)	1 (16.7%)	0 (0%)	0 (0%)	<0.01
Mixed Flowers 1%	3 (50%)	3 (50%)	0 (0%)	0 (0%)	<0.01
Tagetes erecta 2%	3 (50%)	3 (50%)	0 (0%)	0 (0%)	<0.01
Impatiens balsamina (purple) 2%	2 (33.3%)	4 (66.7%)	0 (0%)	0 (0%)	<0.01
Impatiens balsamina (red) 2%	3 (50%)	3 (50%)	0 (0%)	0 (0%)	<0.01
Mixed Flowers 3%	2 (33.3%)	4 (66.7%)	0 (0%)	0 (0%)	<0.01
Tagetes erecta 3%	0 (0%)	2 (33.3%)	4 (66.7%)	0 (0%)	<0.01
Impatiens balsamina (purple) 3%	0 (0%)	1 (16.7%)	5 (83.3%)	0 (0%)	<0.01
Impatiens balsamina (red) 3%	0 (0%)	1 (16.7%)	5 (83.3%)	0 (0%)	<0.01
Mixed Flowers 3%	0 (0%)	2 (33.3%)	4 (66.7%)	0 (0%)	<0.01

Table 5. Results of the organoleptic test on the staining produced from the microscopic examination

Sample	Staining produced from the microscopic examination				Mann whitney test
	1	2	3	4	
Control	0 (0%)	0 (0%)	4 (66.7%)	2 (33.3%)	N/A
Tagetes erecta 1%	5 (83.3%)	1 (16.7%)	0 (0%)	0 (0%)	<0.01
Impatiens balsamina (purple) 1%	5 (83.3%)	1 (16.7%)	0 (0%)	0 (0%)	<0.01
Impatiens balsamina (red) 1%	4 (66.7%)	2 (33.3%)	0 (0%)	0 (0%)	<0.01
Mixed Flowers 1%	5 (83.3%)	1 (16.7%)	0 (0%)	0 (0%)	<0.01
Tagetes erecta 2%	3 (50%)	3 (50%)	0 (0%)	0 (0%)	<0.01
Impatiens balsamina (purple) 2%	2 (33.3%)	4 (66.7%)	0 (0%)	0 (0%)	<0.01
Impatiens balsamina (red) 2%	2 (33.3%)	4 (66.7%)	0 (0%)	0 (0%)	<0.01
Mixed Flowers 3%	1 (16.7%)	4 (66.7%)	1 (16.7%)	0 (0%)	<0.01
Tagetes erecta 3%	0 (0%)	2 (33.3%)	4 (66.7%)	0 (0%)	0.056*
Impatiens balsamina (purple) 3%	0 (0%)	2 (33.3%)	4 (66.7%)	0 (0%)	0.056*
Impatiens balsamina (red) 3%	0 (0%)	2 (33.3%)	4 (66.7%)	0 (0%)	0.056*
Mixed Flowers 3%	0 (0%)	2 (33.3%)	4 (66.7%)	0 (0%)	0.056*

The asterisks (*) accompanying the numbers in the table indicate that the results are not significantly different

Table 6. Detection test on *Ascaris lumbricoides*

Sample		*Ascaris lumbricoides* gold standard		Chi-Square Test
		Positive[a] (N=5)	Negative[b] (N=5)	
Tagetes erecta 1%	Found	5 (100%)	0 (0%)	<0.01*
	Not Found	0 (0%)	5 (100%)	
Impatiens balsamina (purple) 1%	Found	5 (100%)	0 (0%)	<0.01*
	Not Found	0 (0%)	5 (100%)	
Impatiens balsamina (red) 1%	Found	5 (100%)	0 (0%)	<0.01*
	Not Found	0 (0%)	5 (100%)	
Mixed Flowers 1%	Found	5 (100%)	0 (0%)	<0.01*
	Not Found	0 (0%)	5 (100%)	
Tagetes erecta 2%	Found	5 (100%)	0 (0%)	<0.01*
	Not Found	0 (0%)	5 (100%)	
Impatiens balsamina (purple) 2%	Found	5 (100%)	0 (0%)	<0.01*
	Not Found	0 (0%)	5 (100%)	
Impatiens balsamina (red) 2%	Found	5 (100%)	0 (0%)	<0.01*
	Not Found	0 (0%)	5 (100%)	
Mixed Flowers 2%	Found	5 (100%)	0 (0%)	<0.01*
	Not Found	0 (0%)	5 (100%)	
Tagetes erecta 3%	Found	5 (100%)	0 (0%)	<0.01*
	Not Found	0 (0%)	5 (100%)	
Impatiens balsamina (purple) 3%	Found	5 (100%)	0 (0%)	<0.01*
	Not Found	0 (0%)	5 (100%)	
Impatiens balsamina (red) 3%	Found	5 (100%)	0 (0%)	<0.01*
	Not Found	0 (0%)	5 (100%)	
Mixed Flowers 3%	Found	5 (100%)	0 (0%)	<0.01*
	Not Found	0 (0%)	5 (100%)	

*Using Chi-Square Fisher's exact test
[a]Positive means that *Ascaris lumbricoides* eggs were found in the feces
[b]Negative means no *Ascaris lumbricoides* eggs were found in the feces

The T1 and T2 test groups of the four flower dyes showed significant differences ($P < 0.05$) in the number of eggs per gram of feces (EPG) compared to the positive control group, methylene blue, which is known for its effectiveness in detecting parasite eggs. In contrast, the T3 test group with a 3% concentration in all four flower dyes showed no significant difference ($P > 0.05$) compared to the positive control (Figure 1). This result indicates that a 3% concentration of each flower dye matches methylene blue in detecting *Ascaris lumbricoides* eggs, making T3 the optimal concentration for detecting parasite eggs.

3.3. Results of the Identification of Helminth Morphology Test

The morphological examination results of *Ascaris lumbricoides* eggs demonstrate that the dye from *canang* flower waste effectively highlights the three egg layers: the albuminoid layer (in infective eggs), the hyaline layer, and the vitelline layer (Figure 2). In contrast, eggs with damaged walls were excluded from the count, as such eggs are invalid for further analysis (Figure 3).

3.4. Results of pH Analysis and Heavy Metal Contamination Level Test

The results of the pH analysis and heavy metal contamination tests were evaluated against the wastewater quality standards for healthcare facilities as outlined in the Regulation of the Minister of Environment of the Republic of Indonesia Number 5 of 2014, Appendix XLIV, point b. The selection of the 3% concentration treatment (T3) was based on prior experimental findings demonstrating its superior performance. The pH test results in Table 7 indicated good compliance with the gold quality standard across all concentrations of *canang* flower waste extract used in the study. All samples analyzed for heavy metal contamination met the required quality standards (Table 8).

4. Discussion

During organoleptic testing, the 3% *canang* flower waste extract dye solution produced the most favorable results, with a P-value greater than 0.05, indicating effective absorption by cellophane and optimal coloration both in the solution and when applied to glass slides, as well as under microscopy. This result is attributed to the increased solubility of organic dye components at this concentration, which enhances dye performance (Mizuno *et al*. 2024). In detection tests with feces samples containing *Ascaris lumbricoides* eggs, the 3% *canang* flower waste extract dye was as effective as methylene blue. Lower concentrations caused damaged helminth egg structures due to osmotic pressure and were excluded from valid egg counts (Rodgers *et al*. 2021).

The pH analysis showed values within the recommended range of 6-9 for healthcare-related heavy metal contamination, as specified by the Ministry of Environment (2014). Heavy metals pose significant environmental and health risks (Mitra *et al*. 2022). A study conducted by Liu *et al*. (2021) reported that Impatiens balsamina contains lead (Pb) from 4.67 to 421.85 ppm in roots, 2.15 to 208.69 ppm in stems, and 1.24 to 78.74 ppm in leaves. Similarly, *Tagetes erecta* exhibits high levels of heavy metals, with cadmium (Cd) at 346 ppm, zinc (Zn) at 690 ppm, and lead (Pb) at 2.21 ppm in its flower shoots (Madanan *et al*. 2021). However, the 3% flower waste extracts in this study exhibited negligible contamination, which is below the regulatory limits (Indonesian Minister of Environment Regulation No. 5 of 2014, Appendix XLIV, point b). Overall, the *canang* flower waste extract at a 3% concentration shows significant potential as a natural stain for modifying the Kato-Katz technique, providing effective, reliable, and

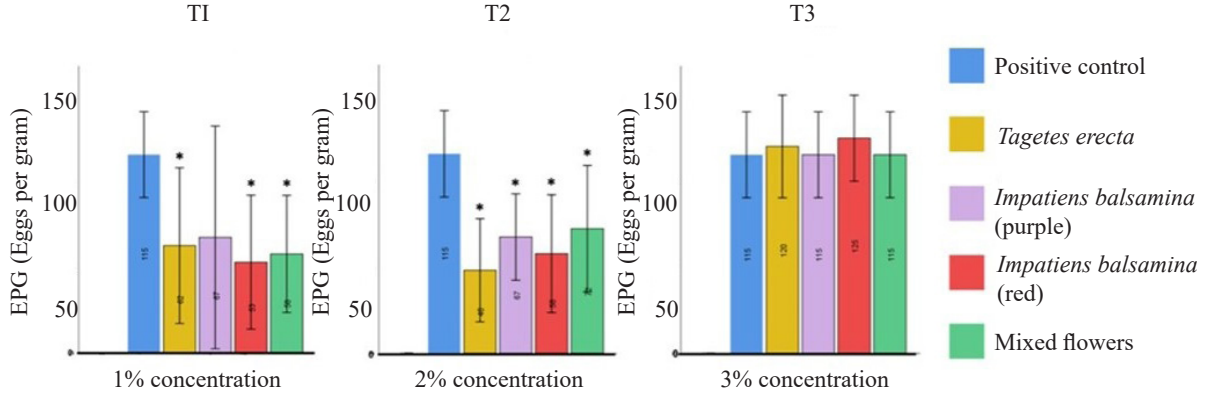

Figure 1. Sub-analysis of the findings on the number of *Ascaris lumbricoides* eggs per gram of feces in the test groups with 1%, 2%, and 3% dye concentrations, using a paired T-test. The asterisk (*) in Figure 1 indicates a significant difference ($P < 0.05$) compared to the positive control

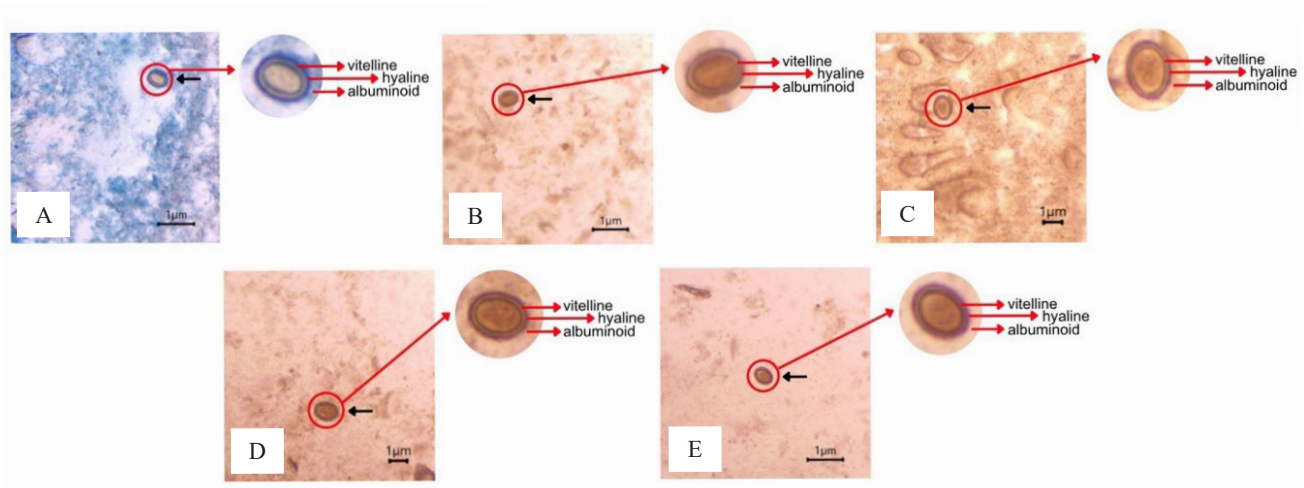

Figure 2. Findings of *Ascaris lumbricoides* eggs (black arrow) using the Kato-Katz method in the T3 treatment group. Figures: A) positive control group, B) T3 group with *Tagetes erecta*, C) T3 group with *Impatiens balsamina* (purple), D) T3 group with *Impatiens balsamina* (red), E) T3 group with mixed flowers

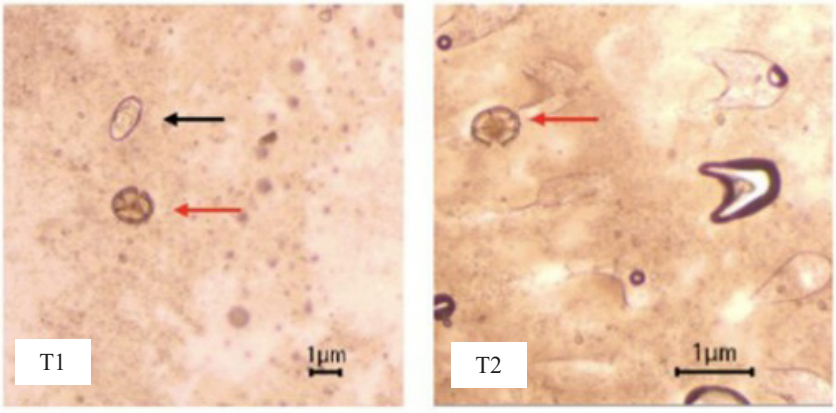

Figure 3. Findings of infertile *Ascaris lumbricoides* eggs with intact walls (black arrow) and *Ascaris lumbricoides* eggs with damaged walls (red arrow). Left to right: T1 group with *Impatiens balsamina* (red), T2 group with *Tagetes erecta*

Table 7. pH analysis result

Treatment	pH			
	Impatiens balsamina (red)	*Impatiens balsamina* (purple)	*Tagetes erecta*	Mixed flowers
T1	6	7	6	6
T2	6	7	7	7
T3	7	7	7	6

*The gold standard pH value for the analysis of heavy metal contamination levels in the healthcare sector is 6-9

Table 8. Heavy metal contamination level test (mg/L)

Sample	pH		Pebble (Pb)		Cadmium (Cd)		Lead (As)		Conclusion
Tagetes erecta 3%	7	6-9	-19.14	0.1	-48.24	0.05	-300.84	0.1	Passing standard
Impatiens balsamina (purple) 3%	7	6-9	-16.98	0.1	-4.29	0.05	-326.94	0.1	Passing standard
Impatiens balsamina (red) 3%	7	6-9	-13.94	0.1	-59.36	0.05	-369.44	0.1	Passing standard
Mixed Flowers 3%	6	6-9	-13.84	0.1	-36.41	0.05	-228.62	0.1	Passing standard

safe results for helminth diagnosis without requiring the separation of flower types.

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