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Color Characteristics of Morning Glory (Ipomoea purpurea) Extract as a Natural Stain for Avian Blood Smear Preparations

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Abstract

Synthetic stains such as Giemsa and Wright remain the standard reagents for blood smear examination, but their repeated use in teaching laboratories raises concerns related to cost, handling safety, and chemical waste. This study evaluated the color characteristics of Morning Glory (Ipomoea purpurea) flower extract as a natural stain for avian blood smear preparations and identified the best-performing tested concentration under the present experimental conditions. A laboratory experiment was conducted using ethanol-macerated Morning Glory extract adjusted to pH 4 and tested at 70%, 80%, and 90%, with unstained smears, methylene blue, and Giemsa as comparators. Blood smears from Columba livia were observed microscopically and evaluated using an ordinal rubric covering clarity, contrast, structural integrity, intensity, and cleanliness, alongside UV-Vis absorbance measurement. The extract showed a maximum wavelength at 641 nm, and absorbance increased with concentration, reaching 2.836 AU at 90%. However, the best microscopic performance among the natural-stain treatments was obtained at 80%, which yielded the highest clarity and contrast scores within the extract group. Kruskal-Wallis analysis indicated significant differences among treatments for clarity ($p = 0.003$) and contrast ($p = 0.001$). Although the Morning Glory extract did not match the performance of methylene blue and Giemsa, the 80% preparation showed pilot-level potential as a greener alternative for teaching laboratories and initial smear observation. These findings should be interpreted cautiously because the study was limited to one avian source, relied primarily on visual scoring, and did not yet include objective image-based analysis or long-term stain-stability testing.

Keywords: Avian Blood Smear; Histological Stain; Ipomoea Purpurea; Morning Glory; Natural Dye

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INTRODUCTION

Blood smear staining is a critical step in avian hematological observation because the stain must provide adequate contrast to distinguish erythrocyte boundaries, nuclear detail, leukocyte morphology, thrombocytes, and background cleanliness under light microscopy (Boes & Durham, 2017; El Othmani et al., 2025; Gulati et al., 2013; Vogelbacher et al., 2024). Synthetic stains such as Giemsa and Wright are widely used because they produce reliable contrast and are familiar in routine laboratory work (Alturkistani et al., 2015; Dibal et al., 2022). However, repeated dependence on synthetic reagents in teaching laboratories is associated with higher recurring cost, handling risk, and chemical waste generation, which has increased interest in safer and lower-waste laboratory practices (Al-Tohamy et al., 2022; Freese et al., 2024; Pandit & Saha, 2025; Romero-Cantú et al., 2025).

Plant-derived pigments have therefore attracted attention as candidate biological stains. Anthocyanins are especially relevant because they are widely available, biodegradable, and highly responsive to solvent composition and pH, all of which influence hue, stability, and adsorption behavior (Khoo et al., 2017; Koop et al., 2022). Morning Glory (Ipomoea purpurea) is known to contain anthocyanin pigments that produce visible coloration under acidic conditions (Baucom et al., 2011; Morita & Hoshino, 2018; Park et al., 2007). However, evidence from pigment chemistry, flower-color biology, or general natural-dye applications cannot automatically be extended to blood smear staining, because smear preparations require not only visible color but also clear cellular boundaries, adequate nucleus-to-background separation, limited residue deposition, and consistent microscopic readability.

Previous studies on natural dyes have addressed several different contexts, including histological preparations (Alshamar et al., 2022; Avwioro et al., 2007; Deepthi et al., 2025; Li et al., 2022; Sachdev et al., 2021; Zhu et al., 2026), chemistry-based pigment characterization (Alegbe & Uthman, 2024; del Hoyo-Meléndez, 2025; Negi, 2025; Szadkowski et al., 2022; Yadav et al., 2023), and textile or material staining applications (Baaka et al., 2023; Hassan et al., 2024; Pizzicato et al., 2023; Rehman et al., 2022), but these contexts are not directly equivalent to avian blood smear evaluation. Previous natural-dye studies have shown that plant extracts may be useful in selected biological preparations (Abubakar & Haque, 2020; Baaka et al., 2023; Bolouri et al., 2022; Godlewska et al., 2021; Kowalczewski & Zembrzuska, 2023; Rehman et al., 2022; Şen & Susurluk, 2026), but the literature remains uneven with respect to smear-specific performance, especially for avian blood. Only a limited number of studies have examined natural pigments specifically for smear-based microscopic preparations (Celedón & Díaz, 2021; Chavan et al., 2025; Parmar & Singh, 2018), and even fewer have focused on avian blood, which presents additional interpretive demands because avian erythrocytes are nucleated (Stier et al., 2013; Yap & Zhang, 2021). Moreover, prior studies have often emphasized visible coloration or general staining feasibility without systematically combining spectrophotometric characterization, side-by-side synthetic comparators, and rubric-based evaluation of smear readability (Jain et al., 2013; Khanvilkar et al., 2025; Pednekar et al., 2021). Thus, the gap addressed in this study lies not in the general availability of plant pigments, but in the limited empirical evidence on whether Morning Glory extract can produce readable avian blood smears under controlled extract concentrations and acidic conditions.

Accordingly, this study examined ethanol-macerated Morning Glory flower extract as a natural stain for *Columba livia* blood smears at three tested concentrations (70%, 80%, and 90%) at pH 4. The study combined UV-Vis characterization with rubric-based microscopic evaluation to determine which tested concentration showed the best performance under the present experimental conditions. The scope of the work was limited to short-term staining performance in one avian source and did not evaluate long-term stability, diagnostic equivalence to routine hematology stains, or cell-type-specific quantitative image analysis.

This work contributes methodologically by combining spectrophotometric characterization with visual scoring and non-parametric statistics to identify the most feasible formula among three extract concentrations at pH 4. The study is also relevant for biology education, where safer and lower-waste alternatives are especially valuable for repeated laboratory practice. Accordingly, the objectives of this study

were to assess the staining characteristics of Morning Glory extract on avian blood smears and to determine the most suitable concentration for use as a natural alternative stain.

METHOD

Research Design

This study employed a laboratory experimental design involving six treatment groups: unstained smear, Morning Glory extract at 70%, Morning Glory extract at 80%, Morning Glory extract at 90%, methylene blue, and Giemsa. The Morning Glory extract treatments were evaluated as the main natural-stain groups, whereas the unstained smear and the two synthetic stains were used as comparison groups. The study was designed to compare staining performance descriptively across five visual parameters and inferentially for the two principal parameters, namely clarity and contrast.

Biological Material

Avian blood smears were prepared from *Columba livia* and used as the biological material in this study. All smears were prepared under the same laboratory workflow to minimize technical variation among treatment groups. Because the present study focused on laboratory-level staining feasibility, the findings should be interpreted as preliminary and limited to the avian source used in this experiment.

Extraction Procedure

Morning Glory flowers were collected and processed to obtain an ethanolic extract as the natural staining material. The extract was prepared by maceration and subsequently adjusted to pH 4 prior to application. The acidic condition was selected to maintain visible anthocyanin expression during staining. Three staining concentrations were prepared from the extract, namely 70%, 80%, and 90%, and each was tested under the same staining workflow.

Preparation of Staining Solutions

The staining solutions consisted of Morning Glory extract at 70%, 80%, and 90%, methylene blue, and Giemsa. The Morning Glory concentrations represented three tested extract levels within the same pH-adjusted preparation system and were compared to determine which concentration provided the best smear readability under the present conditions.

Blood Smear Preparation and Staining Procedure

Blood smears were prepared aseptically by placing one drop of blood on a clean glass slide, spreading it into a thin smear, air-drying, and fixing it in methanol for 5 min. The fixed slides were then exposed to the designated stain treatment, rinsed with distilled water, air-dried, and examined under a binocular microscope at 400× magnification. Positive-control smears were stained with methylene blue and Giemsa following routine laboratory practice. All treatment groups were prepared using the same sequence of smear preparation, fixation, staining, rinsing, drying, and observation to maintain procedural consistency across slides.

Microscopic Observation and Scoring

Microscopic evaluation was conducted using an ordinal scoring rubric that covered five parameters: clarity, contrast, structural integrity, intensity, and cleanliness. Each parameter was scored on a four-point scale, where higher scores

indicated better staining quality. The rubric was used to compare the readability and visual quality of the smear preparations across the six treatment groups, as presented in Table 1.

Table 1. Ordinal scoring rubric used for microscopic evaluation.

Parameter	Score 1	Score 2	Score 3	Score 4
Clarity	Cell boundaries largely unclear	Several structures visible but still blurred	Most cell boundaries visible with moderate sharpness	Cell boundaries clearly distinguished
Contrast	Minimal difference between cells and background	Low contrast, difficult differentiation	Moderate contrast, structures distinguishable	High contrast, structures easily distinguished
Structural integrity	Many cells distorted or disrupted	Some distortion present	Most cells preserved	Cells well preserved with minimal distortion
Intensity	Color too weak for reliable viewing	Weak but visible staining	Adequate staining intensity	Strong but still readable staining
Cleanliness	Background heavily contaminated/precipitated	Noticeable residue or uneven background	Minor residue with acceptable field	Clean background with minimal artifacts

UV-Vis Measurement

The absorbance characteristics of Morning Glory extract were examined using UV-Vis spectrophotometry. The measurement was used to determine the maximum wavelength of the extract and to compare absorbance values among the three tested concentrations. This solution-level characterization was intended to complement the microscopic smear evaluation and to examine whether greater absorbance was associated with better staining readability.

Data Analysis

The data were analyzed descriptively using score profiles across the five staining parameters. Inferential analysis was performed using the Kruskal-Wallis test for the two main parameters, namely clarity and contrast, because the scoring data were ordinal in nature. The interpretation of results emphasized comparative performance among treatments rather than diagnostic equivalence to standard hematological stains.

RESULTS AND DISCUSSION

Absorbance Characteristics of Morning Glory Extract

The spectrophotometric results showed that the Morning Glory extract reached a maximum wavelength at 641 nm across the three tested concentrations. Absorbance increased from 2.604 AU at 70% to 2.677 AU at 80% and 2.836 AU at 90% (Table 2; Figure 1). Under acidic conditions, this visible-region maximum is consistent with the presence of anthocyanin-containing extract, although the present study did not

perform compound-specific profiling. Importantly, the higher absorbance observed at 90% did not automatically translate into better microscopic readability. This indicates that stain performance on blood smears depends not only on pigment density in solution but also on color deposition, residue formation, wash-off behavior, and the optical cleanliness of the smear field.

Table 2. Maximum wavelength and absorbance of Morning Glory extract.

Concentration of Morning Glory extract (%)	Maximum wavelength (nm)	Absorbance at λmax (AU)
70	641	2.604
80	641	2.677
90	641	2.836

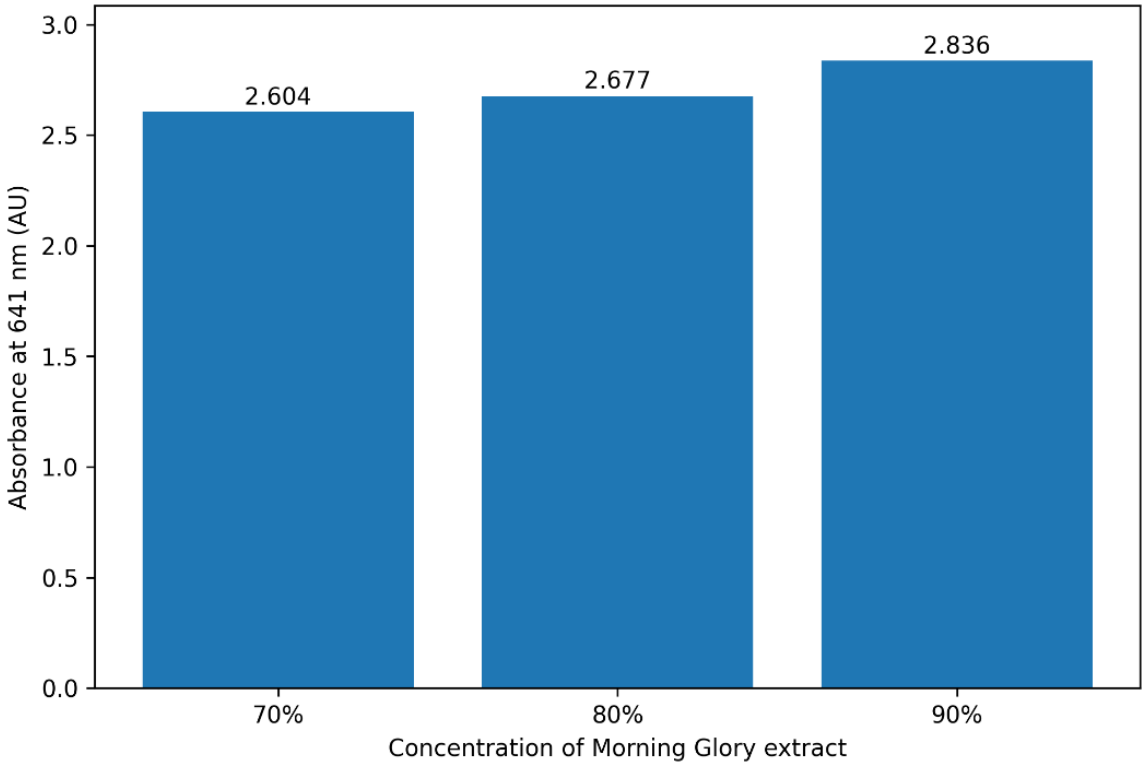

Figure 1. Absorbance comparison of Morning Glory extract measured at 641 nm.

Microscopic Appearance and Visual Quality of Blood Smears

Microscopic observation showed clearer preparations in stained slides than in the unstained smear. The 70% Morning Glory extract improved visibility relative to the unstained preparation, but contrast and edge definition remained limited. The 80% concentration produced the most balanced appearance among the natural-stain treatments, with clearer cell boundaries and more readable contrast. In contrast, the 90% concentration increased color deposition but tended to reduce background neatness and optical balance, suggesting that a more concentrated extract may also increase residue or oversaturation (Figure 2).

The synthetic comparators, methylene blue and Giemsa, still produced the clearest and most contrast-rich micrographs overall. This finding should be interpreted as an important limitation of the present natural extract formulation. Nevertheless, the micrograph series indicates that Morning Glory extract was able to

deposit visible color on avian blood smears and reveal major cellular structures, especially at 80%.

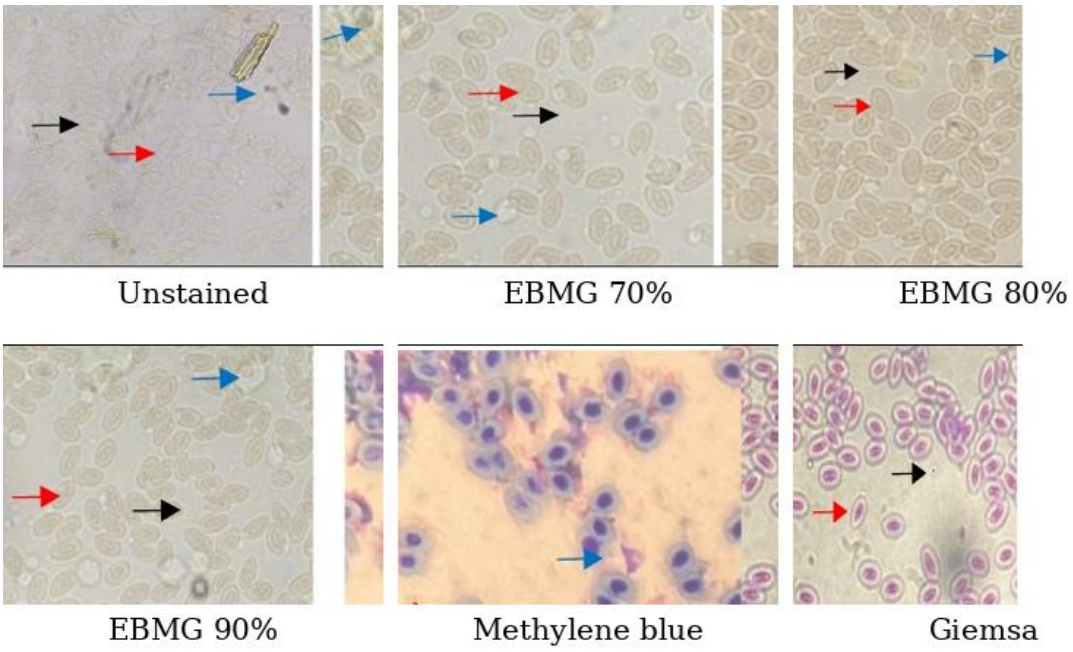

Figure 2. Representative micrographs of avian blood smear preparations under different treatments

The visual scoring profile further supports this interpretation (Figure 3). Across the five descriptive parameters, the 80% extract consistently outperformed the other natural-stain concentrations. It produced the highest natural-stain scores for clarity, contrast, structural integrity, intensity, and cleanliness. By comparison, the 70% treatment was generally too weak, whereas the 90% treatment improved color deposition but remained less balanced than the 80% formulation. Thus, under the present conditions, the 80% concentration appears to provide the best compromise between pigment availability and smear readability.

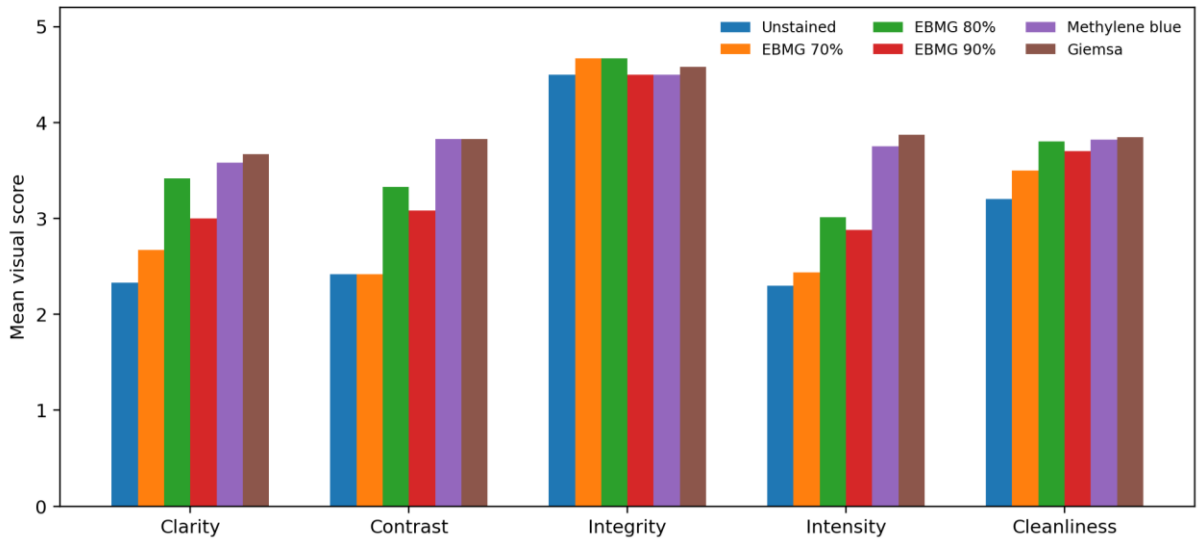

Figure 3. Mean visual quality scores for the six treatment groups across five staining parameters.

Statistical Evidence for the Best-Performing Tested Concentration

The non-parametric analysis confirmed significant differences among treatments for the two principal parameters prioritized for inferential testing. For clarity, the Kruskal-Wallis test yielded p = 0.003, whereas for contrast it yielded p = 0.001 (Table 3). These results support the descriptive pattern showing that the 80% extract performed better than the other Morning Glory concentrations for the two parameters most directly related to smear readability. Because the present manuscript emphasizes overall treatment comparison through Kruskal-Wallis testing, the inferential conclusion should be interpreted at the level of overall group difference rather than detailed pairwise equivalence.

Table 3. Kruskal-Wallis results for clarity and contrast.

Parameter	Test statistic	df	p value
Clarity	17.731	5	0.003
Contrast	20.498	5	0.001

From a staining-chemistry perspective, the superior performance of the 80% extract likely reflects an optimum balance between anthocyanin concentration and smear compatibility (Table 4). When the extract was too dilute, the amount of pigment available for adsorption onto cellular material was insufficient to create strong contrast. When it was too concentrated, the preparation likely retained more background color or residue, reducing the clean optical separation needed for blood smear interpretation. This interpretation is consistent with the known pH sensitivity and stability constraints of anthocyanins, which can alter hue intensity, adsorption behavior, and visual sharpness (Lin et al., 2023; Sharma et al., 2026; West & Mauer, 2013; Xue et al., 2024).

Table 4. Mean clarity and contrast scores for the four core analytical groups.

Treatment group	Clarity mean	Contrast mean
Unstained	2.333	2.415
EBMG 70%	2.667	2.415
EBMG 80%	3.415	3.333
EBMG 90%	3.0	3.083

The findings also show that natural stains should be evaluated in context rather than on pigment intensity alone. The highest absorbance was recorded at 90%, yet the best visual quality was observed at 80%. For smear work, readability depends on an integrated response involving color deposition, transparency, background cleanliness, and preservation of cellular outline. This explains why the strongest solution is not necessarily the most informative microscopically (Bae et al., 2025; Campos-Medina et al., 2024; Kielbassa et al., 2020; Mosiichuk et al., 2023).

Although Morning Glory extract did not exceed the performance of methylene blue or Giemsa, it demonstrated promising educational value as a lower-toxicity and biodegradable alternative. The present results indicate that Morning Glory extract can support preliminary differentiation of avian blood smear components and may be useful for biology teaching laboratories, pilot practical work, or green-laboratory initiatives (Ahmed et al., 2026; Cornish et al., 2021; Reyes et al., 2024; Schell & Bruns, 2024).

The present findings should, however, be interpreted with caution. First, the central outcomes relied primarily on visual scoring, and therefore remain sensitive to observer-related subjectivity. Second, the experiment was conducted under one reported pH condition and three tested extract concentrations only. Third, the blood source was restricted to *Columba livia*, which limits broader biological generalization. Fourth, the study did not yet include long-term stain-stability testing, cell-type-specific quantitative image analysis, or broader validation against routine diagnostic standards. Even with these limitations, the results indicate that Morning Glory extract, particularly at 80%, has preliminary potential for greener teaching-laboratory applications, while still remaining inferior to methylene blue and Giemsa under the present conditions. Future work should therefore focus on improving extract standardization, comparing staining duration and rinsing procedures, testing mordants or co-staining strategies, and integrating objective image-based analysis.

CONCLUSION

Morning Glory flower extract showed measurable potential as a natural stain for avian blood smear preparations. The extract exhibited a maximum wavelength at 641 nm, and absorbance increased with concentration, reaching 2.836 AU at 90%. However, microscopic evaluation demonstrated that the best-performing tested concentration under the present conditions was not the most concentrated solution but the 80% extract at pH 4. The 80% formulation produced the best overall visual performance among the natural-stain treatments and yielded the highest natural-treatment scores for clarity and contrast. Kruskal-Wallis analysis confirmed significant differences among treatments for clarity ($p = 0.003$) and contrast ($p = 0.001$). Although synthetic controls still provided superior image quality, Morning Glory extract can be considered a promising alternative stain for environmentally friendly laboratory use, particularly in teaching and preliminary observational contexts. Nevertheless, broader validation and methodological strengthening are still required before it can be considered a reliable substitute for standard hematological stains.

RECOMMENDATION

Based on the findings of this study, Morning Glory flower extract at 80% concentration and pH 4 is recommended as the most feasible formulation among the tested natural-stain treatments for avian blood smear observation. This formulation provided the best balance between staining intensity, cellular clarity, contrast, structural preservation, and background cleanliness compared with the 70% and 90% extract concentrations. Therefore, the 80% extract may be considered for use in teaching laboratories, introductory hematology practical sessions, and environmentally oriented laboratory activities where the main purpose is to demonstrate general blood smear morphology rather than to perform diagnostic hematological evaluation.

The use of Morning Glory extract should remain limited to preliminary or educational applications until further validation is completed. Although the extract improved the visibility of avian blood smear structures, methylene blue and Giemsa still produced superior microscopic clarity and contrast. For this reason, Morning Glory extract should not yet be recommended as a replacement for standard hematological stains in diagnostic, clinical, or advanced research settings. Instead, it

should be positioned as a promising natural-stain candidate that supports safer, lower-waste, and more accessible laboratory practices.

Future studies are recommended to strengthen the reproducibility and applicability of this staining approach. Further research should evaluate different pH levels, staining durations, rinsing procedures, extract storage conditions, and mordant or co-staining strategies to improve staining consistency. Additional validation using multiple avian species, larger smear replicates, blinded observers, inter-rater reliability testing, and objective image-based analysis would also help determine whether Morning Glory extract can provide more consistent microscopic performance. Compound-specific characterization of the extract is also recommended to clarify the relationship between pigment composition, absorbance profile, and staining quality.

In practical application, laboratories intending to use Morning Glory extract should standardize the extraction procedure, pH adjustment, staining time, and slide evaluation method. Such standardization is necessary to reduce variation between preparations and to ensure that the staining outcome remains interpretable across repeated laboratory sessions.

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Conflict of Interest Statement
Authors state no conflict of interest.

Data Availability
The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions Statement

Name of Author	C	M	So	Va	Fo	I	R	D	O	E	Vi	Su	P	Fu
Sri Wahyuni	✓	✓	✓		✓	✓		✓	✓	✓	✓	✓	✓	
Iin Hindun	✓	✓		✓	✓	✓	✓	✓	✓	✓	✓	✓		
Siti Zaenab	✓	✓		✓	✓	✓	✓	✓		✓	✓	✓		

C : **C**onceptualization
M : **M**ethodology
So : **So**ftware
Va : **Va**lidation
Fo : **Fo**rmal analysis
I : **I**nvestigation
R : **R**esources
D : **D**ata Curation
O : Writing - **O**riginal Draft
E : Writing - Review & **E**diting
Vi : **Vi**sualization
Su : **Su**pervision
P : **P**roject administration
Fu : **Fu**nding acquisition

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