Innovations

Alternatives to Hematoxylin and Eosin Stain in Cytology: Exploring Fruit Ink (Dianella Nigra) and Kumkum

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Abstract

Introduction: *Hematoxylin and eosin (H&E) staining is a gold-standard method in histopathology and cytology, yet its reliance on synthetic dyes raises concerns regarding toxicity, environmental hazards, and high production costs. As a result, the scientific community is exploring sustainable, plant-based alternatives that are safe and cost-effective.* ***Objective:*** *The present study aims to evaluate the cytological staining efficacy of two natural stains—Dianella nigra (New Zealand blueberry) fruit extract as a nuclear stain substituting hematoxylin, and Kumkum, prepared from Curcuma aromatica and slaked lime, as a cytoplasmic counterstain.* ***Materials and Methods:*** *Gingival and buccal smears were collected from healthy individuals, fixed in alcohol, and stained using freshly prepared Dianella nigra and Kumkum solutions. These smears were compared with conventionally stained H&E slides. Staining efficacy was evaluated using four parameters: nuclear contrast, cytoplasmic staining intensity, background clarity, and overall staining quality. Each parameter was scored on a 10-point scale, and statistical analysis was performed using an independent t-test to determine significant differences between the staining methods.* ***Results:*** *The H&E stained slides demonstrated significantly higher mean values across all parameters—nuclear contrast (8.96), cytoplasmic intensity (8.28), background clarity (9.17), and overall staining quality (8.76)—in comparison to the Dianella nigra and Kumkum-stained slides, which scored 5.98, 6.75, 6.30, and 6.33 respectively ($p < 0.001$). However, Kumkum showed promising cytoplasmic contrast and clarity, suggesting its feasibility as a potential natural eosin alternative.* ***Conclusions:*** *Although the natural staining combination did not match the diagnostic clarity of conventional H&E, its eco-friendly, non-toxic properties make it a valuable candidate for cytological applications, especially in resource-constrained or environmentally conscious settings. Further research focusing on optimization, formulation stability, and reproducibility is warranted to enhance its diagnostic reliability.*

Keywords: *Natural stains, Dianella nigra, Kumkum, Hematoxylin alternative, Cytology, Eco-friendly staining, Curcuma aromatica*

Introduction:

Hematoxylin and eosin (H&E) staining is the cornerstone of histopathological and cytological evaluations, providing essential contrast to cellular and tissue structures for accurate diagnosis. The reliance on synthetic dyes raises concerns regarding toxicity, environmental impact, and cost, prompting the exploration of natural staining alternatives.Natural dyes, derived from plant sources, have been investigated for their potential to replace synthetic stains in histology and cytology. Among these, Kumkum—a traditional dye made from Curcuma aromatica (wild turmeric) and slaked lime—has demonstrated efficacy as a counterstain in histological evaluations. Studies have shown that Kumkum can effectively stain oral tissues, providing comparable results to conventional eosin in terms of staining efficacy and the ability to highlight cellular structures [1]. Additionally, Kumkum has been explored as a surrogate for eosin in routine histological sections, with findings indicating its potential to highlight normal structures effectively [2].While specific studies on Dianella nigra (New Zealand blueberry) fruit (Figure 1) extract as a hematoxylin alternative are limited, the broader category of anthocyanin-rich plant extracts has shown promise in staining applications. Anthocyanins, responsible for blue to purple pigmentation in plants, have been utilized in various staining protocols, suggesting potential for Dianella nigra fruit extract in cytological staining.This study examines the viability of using Dianella nigra fruit extract and Kumkum as natural alternatives to traditional H&E staining in cytology, focusing on their staining properties, effectiveness, and potential benefits over synthetic dyes.

Materials and Methods:

Twenty gingival and buccal smears were collected from patients using sterile swabs and fixed in 95% alcohol-filled coupling jars. Kumkum stain was prepared by diluting 15 gm of Kumkum powder in 100 ml of ethanol and mixing it well with a glass rod. Kumkumsolution were stored in a dark chamber covered with foil paper for 48 hours, after which they were filtered for use. Fresh Dianella nigra fruit juice was prepared by crushing 250 gm of the fruit using a mortar and pestle. For staining, the freshly extracted Dianella nigra juice was poured onto the 95% alcohol fixed cytosmears and left for 30 minutes to allow nuclear staining, followed by washing with tap water. Then, the prepared Kumkum stain was applied to the slides for 5 seconds, rinsed gently with running water, and air-dried. Finally, mounting was done using mounting oil to preserve the stained smear for microscopic examination.

Result:

Study Parameter	Study Groups	Mean	S.D	Sig	Mean Diff	95% Confidence Interval	
						Lower	Upper
Nuclear Contrast	**Dianella Nigra**	5.9800	.56921	.000*	-2.98000	-3.38955	-2.57045
	H & E	8.9600	.23664				
Cytoplasmic Staining Intensity	**Dianella Nigra**	6.7500	.36591	.000*	-1.53000	-1.81196	-1.24804
	H & E	8.2800	.21499				
Background and Clarity	**Dianella Nigra**	6.3000	.57542	.000*	-2.87000	-3.27114	-2.46886
	H & E	9.1700	.18288				
Overall Staining Quality	**Dianella Nigra**	6.3300	.32335	.000*	-2.43000	-2.67908	-2.18092
	H & E	8.7600	.18974				

Table 1: Represents The Intergroup Comparison Between The Study Groups By Using The Independent T Test.

The intergroup comparison between the study groups using the independent t-test revealed statistically significant differences across all staining parameters between Dianella nigra and Hematoxylin and Eosin (H&E) staining ($p = 0.000$ for all parameters) (Table -1), (Graph-1).

Figure 1: Ink Fruit (Dianella nigra)

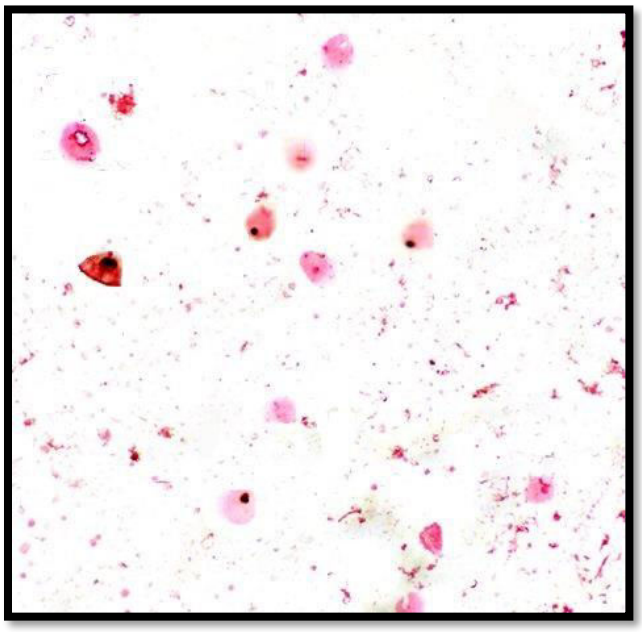
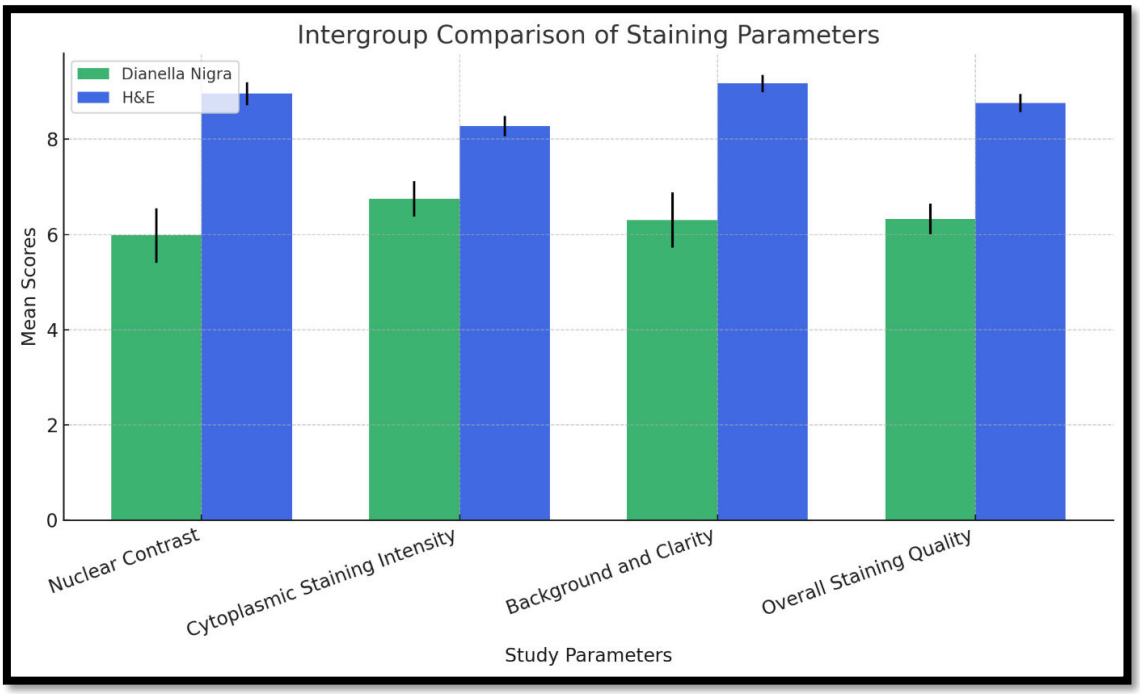


Figure 2: Cytosmear stain of Dianellanigra and Kumkum



Graph 1: The Intergroup Comparison of Staining Parameter

Across all assessed parameters, H&E staining demonstrated significantly better nuclear contrast, cytoplasmic staining intensity, background clarity, and overall staining quality compared to Dianella nigra (Figure 2). The results indicate that while Dianella nigra does provide some level of staining, it is significantly inferior to H&E in terms of contrast, intensity, and clarity.

Parameter	Ink Fruit (Dianella nigra)	Kumkum	Conventional H & E Staining
Nuclear Staining	Deep blue to purple	-	Blue to purple (hematoxylin)
Cytoplasmic Staining	-	Pink to reddish-orange	Pink to red (eosin)
Contrast	Good	Good	Excellent
Uniformity	Consistent staining	Uniform	Highly uniform
Background Staining	Minimal	High	None
Stain Stability	Stable for 48 hours	Stable for 48 hours	Long-term stability
Cytological Clarity	Well-defined nuclear details	Clear cytoplasmic staining	Excellent definition
Environmental Safety	Biodegradable, non-toxic	Non-toxic	Chemical waste concerns

Discussion:

The pursuit of natural alternatives to traditional hematoxylin and eosin (H&E) stains in cytology has intensified due to concerns about the environmental impact and potential health risks associated with synthetic dyes. Recent studies have explored various plant-based extracts for their staining efficacy, aiming to provide safer and eco-friendly options for histopathological examinations [3,4]..On Evaluation, Nuclear Contrast: The mean nuclear contrast was significantly lower in the Dianellanigra group (5.98 ± 0.57) compared to the H&E group (8.96 ± 0.24), with a meandifference of -2.98. The 95% confidence interval ranged from -3.39 to -2.57, confirminga statistically significant reduction in nuclear contrast with Dianella nigra. Cytoplasmic Staining Intensity: The Dianella nigra group showed a lower cytoplasmicstaining intensity (6.75 ± 0.37) compared to the H&E group (8.28 ± 0.21), with a meandifference of -1.53. The 95% confidence interval (-1.81 to -1.25) indicates a significantdifference, suggesting that H&E provides a more intense cytoplasmic stain. Background and Clarity: A significant difference was also observed in backgroundclarity, with Dianella nigra scoring 6.30 ± 0.58 compared to 9.17 ± 0.18 for H&E. Themean difference of -2.87 (95% CI: -3.27 to -2.47) indicates that H&E produces a clearerbackground with better differentiation. Overall Staining Quality: The overall staining quality was significantly lower in theDianella nigra group (6.33 ± 0.32) compared to the H&E group (8.76 ± 0.19), with amean difference of -2.43 (95% CI: -2.68 to -2.18). This suggests that H&E providessuperior overall staining quality.

Curcumin, derived from Curcuma longa (turmeric), has been evaluated as a substitute for eosin. A study compared the staining qualities of curcumin with eosin in H&E procedures and found that curcumin provided comparable results, effectively staining cytoplasmic components without significant differences in staining efficacy [5]. This suggests that curcumin can serve as a viable, biosafe alternative to eosin in histological staining [6].

Anthocyanins, naturally occurring pigments found in various fruits and flowers, have also been explored for their staining properties. Research utilizing anthocyanin extracts from Punica granatum (pomegranate) demonstrated that these pigments could effectively stain histological specimens [7]. Although the staining properties were not as robust as standard dyes, the study highlighted the feasibility of developing eco-friendly, non-toxic, and readily available natural stains for histopathology [8].

A literature review on natural alternatives for chemicals used in histopathology labs discussed various plant-based stains, including those derived from Hibiscus sabdariffa (roselle) and Lawsoniainermis (henna). The review emphasized the potential of these natural dyes to replace synthetic stains, offering benefits such as biodegradability, safety, and cost-effectiveness [9].The use of Dianella nigra (New Zealand blueberry) fruit extract as a nuclear stain and Kumkum as a cytoplasmic stain presents a novel approach in this context. While specific studies on these particular natural dyes are limited, the success of other plant-based stains suggests potential applicability [10,11]. Further research is necessary to evaluate their staining efficacy, stability, and compatibility with existing histological protocols [12].

In conclusion, the shift towards natural staining alternatives in cytology is driven by the need for safer and environmentally sustainable practices. Plant-based dyes like curcumin and anthocyanins have shown promise as substitutes for synthetic stains [13]. The exploration of Dianella nigra fruit extract and Kumkum adds to this growing field, warranting further investigation to establish their utility in routine histopathological applications [14].

Conclusion

Stains extracted from natural fruits and Kumkum helps to avoid hazardless causes by other stains and also these are natural and time consuming .It shows approximately differentiate the cytoplasm and nuclei .These are instant staining .It may be impossible to replace completely the chemical synthesized stains

Declarations

Availability of Supporting Data

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare that they have no competing interests.

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References

1. *Natural stain (Kumkum) formulated by the extract of Curcuma aromatica and slaked lime in histostaining of oral tissues: An observational study. pubmed.ncbi.nlm.nih.gov.*
2. *Efficacy of Kumkum as a Surrogate for Eosin in Routine Histological Sections: An Observational Study. doaj.org.*
3. *Evaluation of biosafe alternative to eosin in hematoxylin and eosin staining procedure: A comparative study. J Oral Maxillofac Pathol. 2023; 27(3):340-344.*
4. *Toward developing natural histologic stains using anthocyanins: A novel approach. J Oral Maxillofac Pathol. 2021; 25(2):340-344.*
5. *The effect of Curcuma longa extract and significance of mordant as an alternative for eosin in H&E: A comparative study. Indian J Pathol Microbiol. 2023; 66(4):697-701.*
6. *Curcuma longa extract - Haldi: A safe, eco-friendly natural cytoplasmic stain. J Oral Maxillofac Pathol. 2017; 21(3):340-344.*
7. *Use of Curcuma longa L. extract to stain various tissue samples for histological studies. Ayu. 2014; 35(4):447-451.*
8. *Natural Alternatives for Chemicals Used in Histopathology Lab - A Literature Review. J Clin Diagn Res. 2016; 10(12):EE01-EE04.*
9. *The wonderful colors of the hematoxylin-eosin stain in diagnostic surgical pathology. Int J Surg Pathol. 2014; 22(1):12-32.*
10. *Hematoxylin and eosin tissue stain in Mohs micrographic surgery: a review. Dermatol Surg. 2011; 37(8):1089-1099.*
11. *Safe Food Coloring Agent as an Alternative to Eosin Stain. Cureus. 2024; 16(9):e69817.*
12. *The potentials of henna leaves extract as counter stain in gram staining reaction. Afr J Microbiol Res. 2010; 4(23):2554-2557.*
13. *Staining of platyhelminthes using aqueous and alcoholic extracts of China rose (Hibiscus rosa-sinensis), sugar beet (Beta vulgaris) and red rose (Rosa hybrida). J Parasit Dis. 2015; 39(4):725-728.*
14. *Alternative staining using distilled water and ethanol extracts of hibiscus (Hibiscus rosa-sinensis) and red beet (Beta vulgaris L.) against Lugol's iodine in wet mount procedure to diagnose ova of intestinal nematodes (Trichuris trichiura and Ascaris lumbricoides). Trop Biomed. 2016; 33(1):1-8.*