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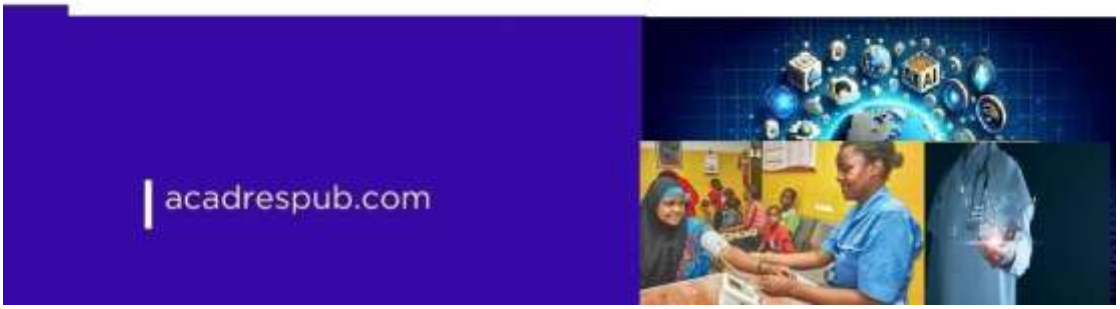

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COMPARATIVE EVALUATION OF ALUM HEMATOXYLIN FORMULATION FOR OPTIMAL HISTOMORPHOLOGICAL VISUALISATION IN COLORECTAL ADENOCARCINOMA

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ABSTRACT

Hematoxylin staining remains fundamental to histopathological diagnosis of colorectal malignancies. Despite extensive use, comparative evaluation of alum hematoxylin formulations on adenocarcinoma specimens remains underexplored. This study evaluated four alum hematoxylin stains: Ehrlich's, Harris's, Cole's, and Gill's on archived colorectal adenocarcinoma tissue to determine optimal stain formulation for nuclear and cytomorphological visualisation. Eight histological sections were prepared from a single established colorectal adenocarcinoma tissue block, with two sections stained using each hematoxylin type. Sections were examined by light microscopy at ×400 magnification, with staining quality assessed by intensity of nuclear uptake, tissue differentiation capability, and morphological clarity. Staining reactions were graded as weakly positive (+), moderately positive (++), strongly positive (+++), or excellently strongly positive (++++). Results revealed Ehrlich's hematoxylin produced excellently strong positive reaction (++++) with superior nuclear and glandular staining, optimal chromatin visualisation, and balanced hematoxylin-eosin colour contrast. Harris's and Cole's hematoxylins produced strongly positive reactions (+++), while Gill's hematoxylin yielded moderately positive staining (++). Ehrlich's formulation demonstrated no background or uneven staining artefacts. These findings establish Ehrlich's alum hematoxylin as the optimal formulation for routine colorectal adenocarcinoma staining, offering superior morphological differentiation critical for histopathological diagnosis and tumour grading. We recommend adoption of Ehrlich's hematoxylin as standard protocol for colorectal cancer tissue processing.

Keywords: Alum hematoxylin; Colorectal adenocarcinoma; Histomorphology; Ehrlich's stain; Stain uptake; Histopathology

Introduction

Colorectal cancer (CRC) ranks as the second most lethal malignancy globally and the third most frequent cancer diagnosis across both sexes(Fan et al., 2021). The histopathological diagnosis of colorectal adenocarcinoma relies fundamentally on light microscopic examination of haematoxylin and eosin (H&E) stained tissue sections(Wang et al., 2021). Despite advances in immunohistochemistry and molecular techniques, alum hematoxylin remains the gold standard nuclear counterstain in diagnostic histopathology, essential for evaluating glandular differentiation, nuclear morphology, and tissue architecture critical for tumour grading(Xu et al., 2024). Hematoxylin is a natural compound derived from the logwood tree (Haematoxylum campechianum), native to Central America. The dye itself is colourless; only its oxidation product, hematein, possesses staining capability(Bai et al., 2023). When combined with metal mordants, typically potassium or ammonium alum hematoxylin forms a cationic dye-mordant complex capable of binding basophilic tissue components, particularly phosphate groups of nucleic acids, producing characteristic blue-purple nuclear coloration(Asadi-Aghbolaghi et al., 2024).

Multiple alum hematoxylin formulations have been developed, including Ehrlich's, Harris's, Cole's, Gill's (variants I, II, III), Mayer's, and Carazzi's. Each formulation differs in oxidation method, mordant concentration, additives, and staining kinetics(Sasaki et al., 2022). Currently, no consensus exists regarding optimal alum hematoxylin formulation specifically for colorectal adenocarcinoma diagnosis(Bukhari et al., 2020). This study comparatively evaluated four alum hematoxylin stains on colorectal cancer tissue to determine which formulation provides superior histomorphological visualisation, optimal nuclear clarity, and minimal staining artefacts.

Aim of the Study

This study aims to compare four alum hematoxylin formulations (Ehrlich's, Harris's, Cole's, and Gill's) on colorectal adenocarcinoma tissue to identify which provides optimal stain uptake, superior nuclear morphological visualisation, and clearest tissue differentiation for diagnostic histopathology.

Materials and Methods

Study Design and Specimen

This comparative analytical study was conducted using an archived, established colorectal adenocarcinoma tissue block retrieved from the Histopathology Unit, Igbinedion University Teaching Hospital, Okada, Edo State, Nigeria. A total of eight histological sections were prepared from this single tissue block, with two sections stained using each of four alum hematoxylin formulations, ensuring identical tissue substrate for comparative evaluation.

Inclusion and Exclusion Criteria

Inclusion criteria: Established colorectal adenocarcinoma tissue block with confirmed histopathological diagnosis.
Exclusion criteria: All other malignant tumour types, normal colon tissue, and non-cancerous colon pathology were excluded to maintain homogeneity.

Ethical Approval

Ethical clearance was obtained from the Research Ethics Committee of the Igbinedion University Teaching Hospital Okada Edo State, Nigeria (IUTH/R.23/VOL1/43) prior to conduct of this study.

Tissue Sectioning

Serial tissue sections of 5 µm thickness were prepared from the paraffin-embedded colorectal adenocarcinoma tissue block using a Leica semi-automatic microtome (Leica Microsystems, Wetzlar, Germany). Sections were mounted on labelled glass microscope slides and allowed to dry at room temperature.

Preparation of Hematoxylin Staining Solutions

Ehrlich's Hematoxylin: 2 g hematoxylin was dissolved in 100 mL absolute ethanol. This was combined with 100 mL distilled water, 10 mL glacial acetic acid, and 15 g potassium alum with constant stirring. 100 mL glycerin was added to stabilise the solution and prolong shelf life. Natural ripening was achieved through exposure to sunlight for approximately 2 months.

Harris's Hematoxylin: 2.5 g hematoxylin was dissolved in 25 mL absolute ethanol and added to 50 g potassium alum dissolved in 500 mL warm distilled water. The mixture was brought to rapid boil, and 0.5 g sodium iodate was carefully added. The flask was plunged into ice-cold water for rapid cooling. Upon cooling, 20 mL glacial acetic acid was added. The solution was ready for immediate use.

Cole's Hematoxylin: Constituents included 1.5 g hematoxylin, 50 mL 1% iodine in 95% ethanol, 700 mL

saturated aqueous potassium alum, and 250 mL distilled water. Distilled water was heated to boiling, hematoxylin dissolved, iodine and alum added, then cooled and filtered. Shelf life of approximately 3 months.

Gill's Hematoxylin: Constituents included 730 mL distilled water, 250 mL ethylene glycol, 2 g hematoxylin, 0.2 g sodium iodate, 17.6 g aluminium sulphate, and 20 mL glacial acetic acid. Reagents were dissolved in order and mixed for 1 hour at room temperature. Ready for immediate use.

Staining Procedure

All sections underwent standard deparaffinisation and rehydration: three 2-minute changes of xylene, two changes of 100% ethanol (5 and 3 minutes), 95% ethanol (2 minutes), and distilled water (2 minutes). Sections were then stained in respective hematoxylin solutions: Ehrlich's (15 minutes), Harris's (2-5 minutes), Cole's (10 minutes), and Gill's (10 minutes). All slides were rinsed in distilled water, differentiated with acid alcohol (10 dips), rinsed again, and blued using Scott's tap water substitute (1 minute). Counterstaining with 1% eosin Y was performed for 1-2 minutes, followed by dehydration in 95% and 100% ethanol (2 changes each), clearance in xylene (2 changes, 2 minutes each), and mounting with cover glass and mounting medium.

Microscopic Examination and Evaluation

Histological sections were examined using a light microscope (Swift Binocular Microscope with integrated illumination system, Nikon Opticshot-2, Tokyo, Japan) at ×400 magnification. Staining quality was assessed using the following criteria: (1) intensity of nuclear stain uptake, (2) clarity of chromatin and nucleolar detail, (3) extent of background staining, (4) evenness of staining distribution, and (5) hematoxylin-eosin colour balance. Staining reactions were graded as: weakly positive (+), moderately positive (++), strongly positive (+++), or excellently strongly positive (+++). Morphological markers evaluated included malignant glandular cells, blood cells, and stromal components.

Results

Staining results were consistent across sections prepared from the same tissue block, with each hematoxylin formulation maintaining its characteristic staining pattern. Results demonstrated variable staining quality dependent upon hematoxylin type employed.

Ehrlich's Hematoxylin

Sections stained with Ehrlich's hematoxylin demonstrated excellently strong positive reaction (+++++) with superior nuclear staining intensity. Malignant glandular cells displayed distinct blue-purple nuclear coloration with excellent chromatin and nucleolar detail visualisation. Lamina propria and stromal components were clearly delineated. The hematoxylin-eosin colour balance was optimal, with eosinophilic structures displaying appropriate pink-red coloration without overwhelming nuclear detail. No background staining or uneven staining artefacts were observed.

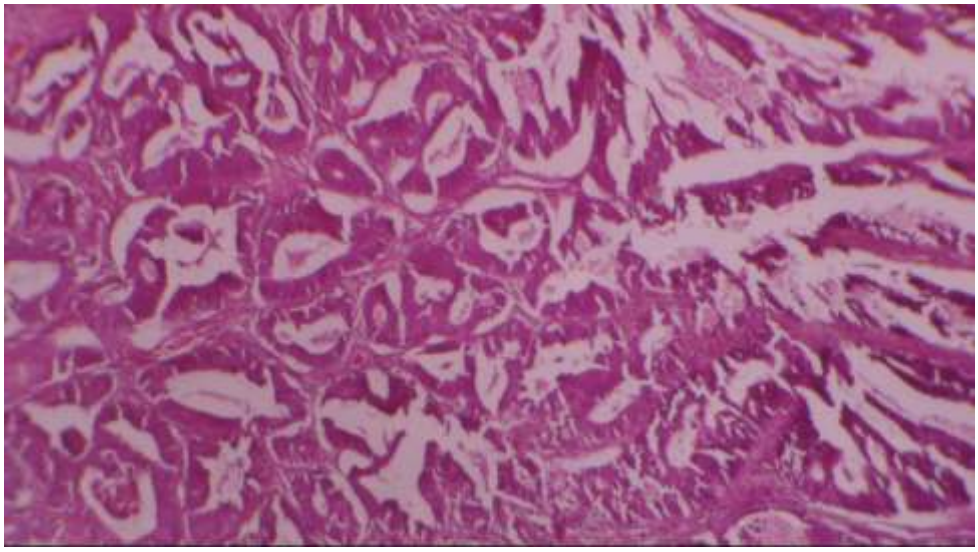

Plate 1. Colonic cancer tissue stained with ERLICH HX showing: excellently strongly positive reaction (+++) on glandular cells: long arrow, lamina propria (short arrow) (H&E x 40)

Harris's Hematoxylin

Harris's hematoxylin produced strongly positive reactions (+++) on malignant glands, blood cells, and submucosa. Nuclear staining was pronounced and allowed clear differentiation of abnormal morphological indices. Chromatin detail was visible, though slightly less crisp than Ehrlich's staining. Minor background staining was noted. The stain provided adequate visualisation for diagnostic assessment.

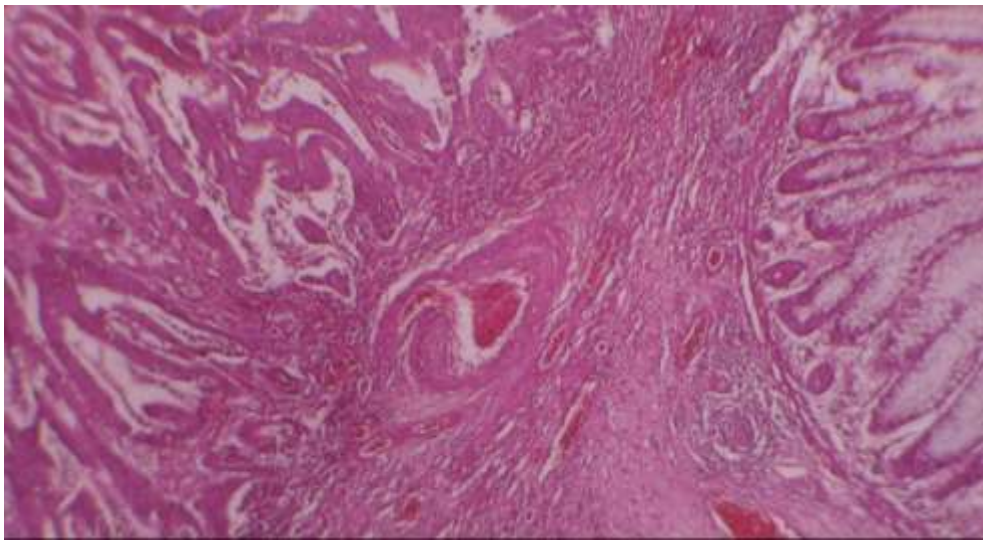

Plate 2. Colonic cancer tissue stained with HARRIS DYE showing: strongly positive reaction (+++) on malignant glands: long arrow, red blood cells: short arrow, submucosa: arrow head (H&E x 40)

Cole's Hematoxylin

Cole's hematoxylin yielded strongly positive reactions (+++) on malignant glands, stromal components, and muscularis mucosa. Nuclear staining was intense and allowed identification of cellular components. However, occasional oxidative precipitate deposits were observed on slides. Overall staining quality was good but slightly inferior to Ehrlich's.

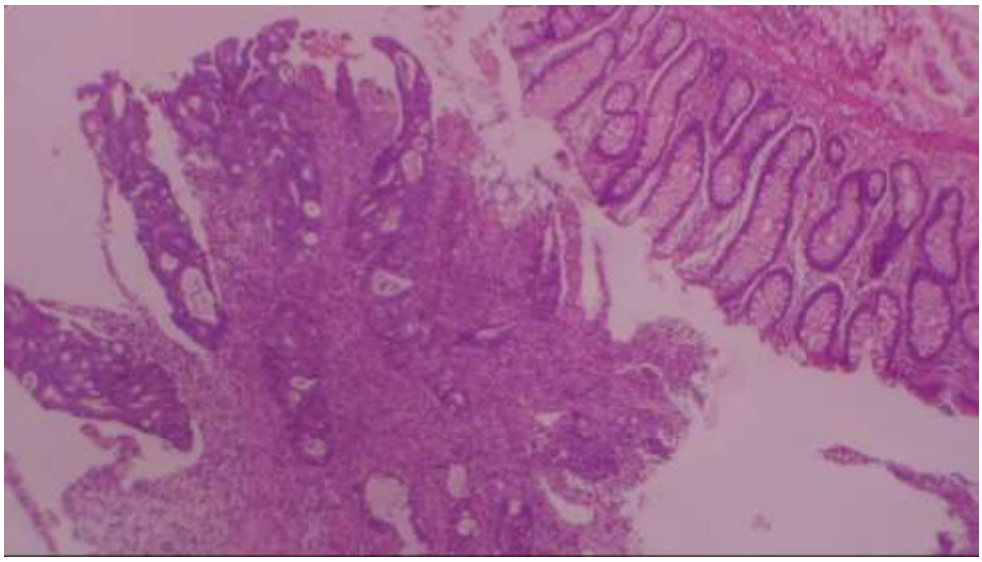

Plate 3...Colonic cancer tissue stained with COLLES DYE showing: strongly positive reaction (+++) on malignant glands: long arrow, stroma: short arrow, muscularis mucosa: arrow head (H&E x 40)

Gill's Hematoxylin

Gill's hematoxylin demonstrated moderately positive reactions (++) on malignant glands and lamina propria. Nuclear staining intensity was reduced compared to other formulations, resulting in weaker chromatin and nucleolar visualisation. Although tissue differentiation was possible, the pale nuclear coloration rendered nuclear detail less distinct. Overall diagnostic utility was compromised.

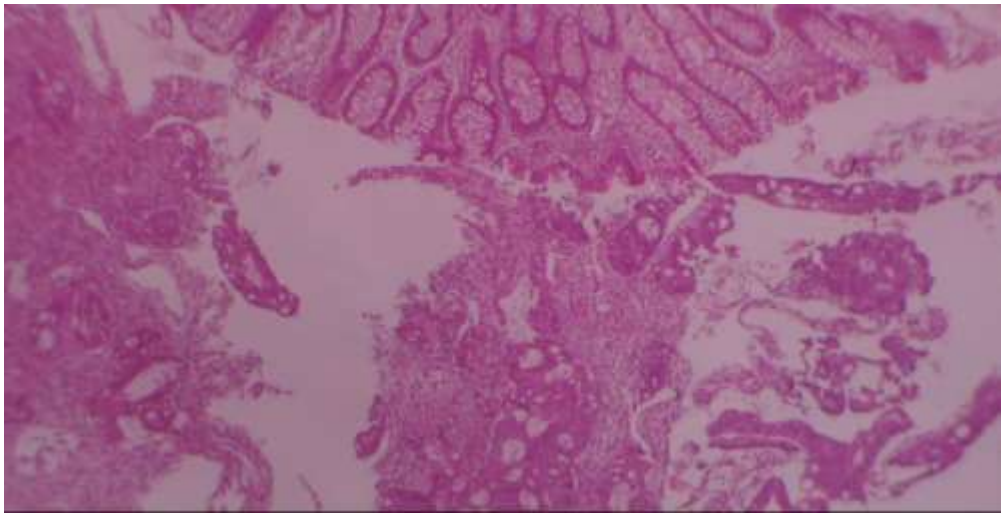

Plate 4. Colonic cancer tissue stained with GILLS DYE showing: moderately positive reaction (++) on malignant glands: long arrow, laminar propria: short arrow (H&E x 40)

Table 1. Comparative Staining Characteristics of Alum Hematoxylin Formulations on Colorectal Adenocarcinoma

Hematoxylin Type	Staining Reaction	Key Features	Diagnostic Utility
Ehrlich's	++++ (Excellent)	Superior nuclear intensity; excellent chromatin detail; optimal H&E balance; no artefacts	Optimal for routine colorectal cancer diagnosis
Harris's	+++ (Strong)	Good nuclear staining; adequate chromatin visibility; minor background staining	Acceptable secondary option
Cole's	+++ (Strong)	Intense nuclear staining; oxidative precipitate deposits present	Acceptable with artefact limitation
Gill's	++ (Moderate)	Weak nuclear intensity; reduced chromatin detail; pale coloration	Suboptimal; not recommended

Discussion

The present study demonstrated that Ehrlich's alum hematoxylin formulation provides superior histomorphological visualisation of colorectal adenocarcinoma compared to Harris's, Cole's, and Gill's formulations. The excellence of Ehrlich's performance likely derives from multiple factors inherent to this formulation.

Ehrlich's formulation incorporates 15 g potassium alum per 100 mL solution, providing optimal mordant concentration for stable dye-mordant complex formation. The inclusion of 100 mL glycerin serves dual functions: stabilising the solution against oxidative degradation and reducing evaporation rates, thereby maintaining staining consistency(Hamdy & Hassabo, 2021). The acidic component (glacial acetic acid) optimises pH for selective nuclear staining whilst preventing over-staining of cytoplasmic components. Natural ripening via sunlight exposure ensures gradual, controlled oxidation of hematoxylin to hematein, eliminating risks of oxidative over-products that compromise staining quality and produce precipitates(Chlipala et al., 2020; Kim et al., 2023).

Conversely, Gill's formulation, whilst incorporating similar alum mordant concentration (17.6 g aluminium sulphate), produced inferior staining. This may reflect differences in solvent composition substitution of ethylene glycol for traditional alcohol which may affect dye solubility, uptake kinetics, or tissue penetration(Dawood et al., 2021). Additionally, Gill's shorter ripening period (immediate use vs. 2-month natural ripening in Ehrlich's) may result in incomplete hematoxylin oxidation and reduced hematein availability(Dunn et al., 2024; Li et al., 2024).

Harris's formulation utilises mercuric oxide-induced rapid oxidation, permitting immediate use. However, rapid oxidation carries risks of oxidative over-products, potentially explaining slightly inferior chromatin detail clarity. Cole's formulation, despite incorporating iodine as oxidant, exhibited occasional precipitate deposits visible artefacts that reduce diagnostic confidence(Sasaki et al., 2022; Yao et al., 2021).

The superiority of Ehrlich's hematoxylin for colorectal adenocarcinoma aligns with previous literature reporting excellent performance of Ehrlich's formulation on acid-decalcified bone and cartilage, and confirmed by contemporary studies evaluating hematoxylin subtypes in Mohs micrographic surgery for cutaneous malignancies. This study extends these findings to gastrointestinal malignancy(Gaytán et al., 2020; Morrison et al., 2021).

Limitations of this study include use of single tissue block (restricting generalisation across adenocarcinoma grades and histological subtypes) and absence of quantitative staining intensity measurement via digital image analysis. Future studies employing multiple specimens across adenocarcinoma differentiation grades and utilising objective densitometry would strengthen findings.

Conclusion

This comparative evaluation establishes Ehrlich's alum hematoxylin as the optimal formulation for histopathological staining of colorectal adenocarcinoma. Superior nuclear staining intensity, excellent chromatin and nucleolar detail visualisation, balanced hematoxylin-eosin colour equilibrium, and absence of staining artefacts collectively position Ehrlich's as the formulation of choice for routine diagnostic histopathology of colorectal cancer. Harris's and Cole's formulations provide adequate diagnostic utility as secondary options. Gill's hematoxylin provides suboptimal staining unsuitable as primary choice.

We recommend adoption of Ehrlich's hematoxylin as standardised protocol in histopathology laboratories processing colorectal cancer specimens. Further comparative studies on normal and benign colorectal tissue would complement present findings. Prospective studies evaluating Ehrlich's performance across varied histological grades and subtypes of adenocarcinoma are warranted.

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