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Full Length Research

Evaluation of cashew apple (*Anacardium occidentale L.*) aqueous extract as a natural histological counterstain in rat tissues

Barde Israel Joshua[1,3]*, Ishaku Leo Elisha[1], Shekaro Audu[1], Ashem Madi Lamba[2], Umoru Mada Alesa[1], Ugbe Aondona Dominic[1], Choji Tobias Peter Pwajok[1], Ngulukun Samuel Sati[1], Dashe Yakubu Gunya[1], Masagbor Otimize Yvonne[3], Lev Kolodzieyski[3] and Zhang Maodong[3]

[1]National Veterinary Research Institute (NVRI), Vom, Plateau State, Nigeria.
[2]Federal College of Animal Health and Production Technology (FCAHPT), Vom, Plateau State, Nigeria.
[3]International Toxicology Research Laboratory, Baie-D'Urfé, Montreal, Quebec, Canada.

*Corresponding author. Email: jbarde@itrlab.com

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ABSTRACT: Staining histological sections prior to microscopic examination is an essential diagnostic technique in histopathology to expose fine cellular microarchitecture. Standard Hematoxylin and Eosin (H&E) staining relies heavily on synthetic eosin as a cytoplasmic counterstain; however, commercial eosin is costly, non-biodegradable, and presents potential health hazards to laboratory personnel. This exploratory pilot study evaluated the potential of an aqueous extract derived from cashew fruit (*Anacardium occidentale* L.) as a natural, plant-based substitute for synthetic eosin in rat tissues. Fresh cashew apples were dried at 30°C for 14 days, pulverised, and extracted in distilled water (10% w/v) for 24 hours, yielding a concentrated extract with a measured pH of 4.6. Staining formulations were prepared across five extract masses (0.15, 0.3, 1.0, 1.5, and 2.0 g) and evaluated under three chemical modifications: accentuated, unaccentuated (ammonia and alcoholic iodine), and modified with 0.4 mL glycoacetic acid. Tissue sections from five organs (intestine, kidney, lung, liver, and skin; harvested from 4 adult albino rats, 20 tissue blocks total) were counterstained at durations of 5, 10, and 20 minutes alongside standard H&E controls. Qualitative visual assessment by two blinded assessors indicated that cashew fruit extract produced readable cytoplasmic counterstaining with a silver-to-brownish pink hue, exhibiting clear nucleo-cytoplasmic contrast against hematoxylin. Formulations modified with glycoacetic acid (1.0 g and 2.0 g) achieved rapid cytoplasmic staining within 5 to 10 minutes, yielding visual clarity comparable to routine H&E controls. Intestine, lung, liver, and skin demonstrated vivid stain absorption, whereas kidney sections showed lower relative intensity. While these preliminary findings demonstrate the feasibility of cashew apple extract as a natural counterstain, formal toxicity, cost-effectiveness, and quantitative equivalence were not evaluated in this pilot study.

Keywords: *Anacardium occidentale*, cashew apple extract, cytoplasmic counterstain, natural dyes, ethnohistology, rat tissue, qualitative evaluation.

INTRODUCTION

Histopathological examination relies fundamentally on differential staining of tissue sections to accentuate cellular morphology, architectural boundaries, and pathological alterations under light microscopy. For over a century, the combination of Hematoxylin and Eosin (H&E) has served as the gold standard routine staining system globally. Hematoxylin acts as a basic dye with high affinity for acidic nuclear structures (DNA and RNA), whereas synthetic eosin functions as an acidic counterstain that selectively binds basic cytoplasmic proteins, imparting a distinctive

pink-to-red colouration (Dunn *et al*., 2024).

Despite its ubiquitous utility, synthetic eosin presents substantial economic, environmental, and occupational health concerns. Synthetic dyes are non-biodegradable chemical reagents whose continuous handling exposes pathologists, technicians, and researchers to chronic toxicity risks and chemical hazards (Barde *et al*., 2021). Furthermore, the rising commercial cost and logistical supply constraints of imported chemical stains pose operational challenges for diagnostic and research laboratories in developing countries. Consequently, there is a growing imperative to identify natural, biodegradable, non-hazardous, and cost-effective plant-derived pigments as sustainable alternatives to synthetic dyes.

The cashew tree (*Anacardium occidentale* L.), a member of the Anacardiaceae family, is a drought-resistant tropical crop extensively cultivated across 27 states in Nigeria, yielding over 2.7 million metric tonnes globally (FAO, 2013; Olife *et al*., 2017). While cashew nuts are harvested for international trade, the succulent pseudo-fruit—commonly termed the cashew apple—represents a massive agricultural byproduct that remains largely underutilised (Ologunde *et al*., 2017; Asogwa *et al*., 2008; Adeigbe *et al*., 2019). The cashew apple contains rich phytochemical profiles, including polyphenols, tannins, organic acids, and natural pigments (Akinhanmi *et al*., 2008).

Although several botanical extracts—such as *Lawsonia inermis* (henna), *Hibiscus sabdariffa* (roselle), *Curcuma longa* (turmeric), and *Pterocarpus osun* have been explored as alternative histological stains (Avwioro *et al*., 2007; Avwioro *et al*., 2005; Adobu *et al*., 2026; Barde *et al*., 2021), significant research gaps remain regarding the utility of fruit waste extracts as functional cytoplasmic counterstains across diverse organ architectures. Previous cashew research has focused primarily on nut shell liquid and seed processing (Akinhanmi *et al*., 2008; Adeigbe *et al*., 2019), leaving the histopathological staining potential of the cashew apple largely uninvestigated. Preliminary physicochemical testing revealed that aqueous cashew fruit extract possesses an acidic pH of 4.6. This acidic pH motivated our evaluation of the extract as a potential cytoplasmic counterstain, under the hypothesis that its inherent acidity facilitates electrostatic binding to basic cytoplasmic proteins. Therefore, this exploratory pilot study aimed to evaluate the staining properties of aqueous cashew apple extract across five rat tissues, examining the effects of extract concentration, mordanting, chemical modification with glycoacetic acid, and staining exposure duration.

MATERIALS AND METHODS

Study area and laboratory locations

Plant material extraction and staining solution formulations were conducted at the Biochemistry Laboratory of the Federal College of Animal Health and Production Technology (FCAHPT), Vom. Animal tissue processing, microtomy, staining trials, and microscopic evaluations were performed at the Histopathology Laboratory, Central Diagnostic Laboratory, National Veterinary Research Institute (NVRI), Vom, Plateau State, Nigeria (latitude 9°50' to 10°00' N, longitude 8°55' E, elevation ~1,290 m above sea level; James *et al*., 2024).

Plant material collection, processing, and aqueous extraction

Fresh, mature cashew (*Anacardium occidentale* L.) fruits were collected locally, thoroughly washed with distilled water to remove superficial debris, and processed following procedures described by Avwioro *et al*. (2005). The fruits were oven-dried at 30°C for 14 days and subsequently pulverised into coarse powder using a mechanical laboratory mill. Extraction was performed according to the aqueous extraction protocol outlined by Avwioro *et al*. (2005) with specified modifications. Briefly, 100 g of the dried cashew fruit powder was infused in 1000 mL of distilled water (10% w/v) at room temperature (25 ± 2°C) for 24 h. The mixture was filtered through Whatman No. 1 filter paper, and the filtrate was concentrated by gentle evaporation on a thermostatically controlled hot plate at 60°C until a dry residue was obtained, yielding approximately 18.5 g of dried extract residue per 100 g of starting powder (18.5% w/w extraction yield). The dried extract was scraped, weighed, reconstituted in distilled water at specified working concentrations, and its pH was measured using a calibrated digital pH meter (Model HI2211, Hanna Instruments, Woonsocket, RI, USA), recording a pH of 4.6 at 25°C. Reconstituted stock solutions were stored in airtight amber glass bottles at 4°C until use.

Ethical approval and animal source

Ethical approval for animal handling and tissue collection was granted by the Animal Use and Care Committee (AUCC) of the National Veterinary Research Institute (NVRI), Vom, Nigeria (Approval Ref No: NVRI/AUCC/2026/014). Four (4) healthy adult albino rats (*Rattus norvegicus*, 2 males and 2 females, 12–14 weeks old, weighing 180–220 g) were sourced from the Experimental Animal Section of NVRI, Vom. A sample size of 4 rats (yielding 20 tissue blocks across 5 organs) was selected for this exploratory pilot study to evaluate staining consistency across diverse organ systems while adhering to animal reduction guidelines (3Rs).

Euthanasia and tissue harvesting

Animals were humanely euthanised by intraperitoneal overdose of sodium pentobarbital (150 mg/kg) in strict compliance with the American Veterinary Medical

Association (AVMA) Guidelines for the Euthanasia of Animals (AVMA, 2020). Five organ tissues—intestine, kidney, lung, liver, and skin were immediately harvested from each animal (20 tissue blocks total) (Raheem *et al*., 2015).

Histological processing

Tissue specimens were fixed in 10% neutral buffered formalin for 24–48 h, dehydrated through an ascending ethanol series (70%, 80%, 90%, 95%, and 100%), cleared in xylene, and embedded in paraffin wax blocks. Thin sections (4µm thick) were cut using a rotary microtome (Leica RM2125 RTS, Leica Biosystems, Wetzlar, Germany) and mounted on clean glass slides.

Staining formulations and protocol

Reagents and chemicals

Reagents utilised included Cole's Hematoxylin primary nuclear stain, commercial 1% synthetic Eosin Y control (BDH Chemicals, Poole, UK), Ammonia solution 28%, Iodine crystals, potassium iodide, Ethanol 95% (Merck, Darmstadt, Germany), Glycoacetic acid (glacial acetic acid and glycerol mixture; Sigma-Aldrich, St. Louis, MO, USA), and DPX mounting medium (BDH Chemicals, UK).

Experimental staining groups

Staining solutions were prepared by adapting Cole's Hematoxylin method parameters (Avwioro *et al*., 2007), replacing commercial synthetic eosin with cashew apple extract across five concentrations (0.15, 0.3, 1.0, 1.5, and 2.0 g). Seventeen (17) distinct experimental figure conditions were established across four primary formulation groups:

Control group (H&E): Primary nuclear staining with Cole's Hematoxylin (5 min) followed by standard synthetic 1% Eosin Y counterstaining for 5 min.

Unmordanted extract: Cashew extract reconstituted in distilled water (0.15, 0.3, and 1.5 g extract) without chemical additives; stained for 10 min and 20 min.

Mordanted extract: Cashew extract (0.15, 0.3, and 1.5 g) combined with an alkaline-iodine additive mixture (140 mL ammonia water + 10 mL 1% iodine in 95% ethanol); stained for 10 min and 20 min. (Note: Ammonia-alcoholic iodine served as an alkaline/oxidizing additive mixture to facilitate dye retention).

Glycoacetic acid modified: Cashew extract (1.0 g and 2.0 g) modified with 0.4 mL glycoacetic acid, 320 mL 95% ethanol, and 50 mL distilled water; stained for 5 min and 10 min.

Staining protocol: Tissue slides were dewaxed in xylene (2 changes, 5 min each), rehydrated through descending ethanol grades to water, and stained with Cole's Hematoxylin for 5 min. Slides were rinsed in tap water, differentiated in 1% acid alcohol, blued in running tap water for 10 min, and counterstained with the respective cashew extract formulations or control eosin. After counterstaining, slides were rapidly dehydrated in absolute ethanol, cleared in xylene, and mounted with DPX synthetic resin.

Microscopic examination and qualitative evaluation rubric

Microscopic equipment

Stained tissue sections were examined under a high-resolution light microscope (Olympus BX53, Olympus Corp., Tokyo, Japan) equipped with an Olympus DP74 digital camera. Photomicrographs were acquired at objective magnifications of ×100 (broad architectural field for intestine, lung, skin) and ×400 (cellular detail for kidney, liver) under standardised light intensity and exposure parameters. Calibrated micron-scale bars (100 µm for ×100; 25 µm for ×400) were embedded into composite figure panels.

Qualitative evaluation rubric

To minimise subjectivity, slides were evaluated independently by two experienced histopathologists blinded to the slide treatment codes. Staining performance was assessed across four qualitative parameters:

1. Cytoplasmic Stain Intensity (0 = None, 1 = Faint/Weak, 2 = Moderate, 3 = Strong/Vivid)
2. Nucleo-Cytoplasmic Contrast (0 = Poor/Obscured, 1 = Fair, 2 = Good, 3 = Excellent)
3. Organ Architectural Definition (0 = Distorted, 1 = Fair, 2 = Clear, 3 = High Definition)
4. Background Cleanliness (0 = Heavy Precipitation/ Muddy, 1 = Moderate Background, 2 = Clean)

Statistical analysis

Given the exploratory pilot design of this study, data analysis was limited to qualitative visual comparisons and descriptive scoring. No formal statistical hypothesis testing was performed.

RESULTS

Physicochemical characteristics of cashew fruit extract

The aqueous extraction of dried cashew apple yielded a

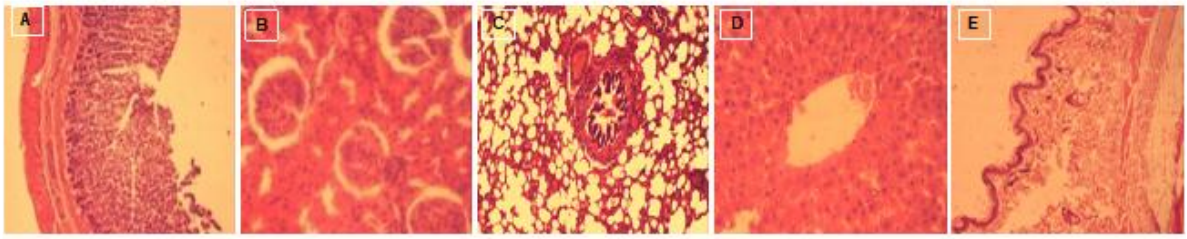


Figure 1. Photomicrographs of albino rat tissues stained with standard Hematoxylin and Eosin (H&E) control. A. Intestine section showing mucosal histology (×100). B. Kidney section (×400). C. Lung section showing alveolar microarchitecture (×100). D. Liver section showing parenchymal structure (×400). E. Skin section (×100).



Figure 2. Photomicrographs of rat tissues counterstained with 0.3 g aqueous cashew fruit extract with ammonia-iodine additive for 10 minutes. A. Intestine (×400). B. Kidney (×400). C. Lung (×400). D. Liver (×400). E. Skin (×100).

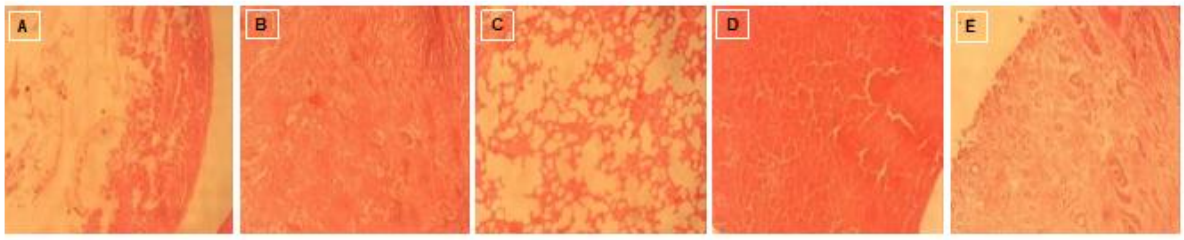


Figure 3. Photomicrographs of rat tissues counterstained with 0.3 g aqueous cashew fruit extract with ammonia-iodine additive for 20 minutes. A. Intestine (×100). B. Kidney (×400). C. Lung (×100). D. Liver (×100). E. Skin (×100).

dark, concentrated extract with a measured acidic pH of 4.6. This measured acidic pH provides hydrogen ions that favour electrostatic interaction with basic amino acid residues on cytoplasmic proteins, providing the physicochemical basis for its function as an acidic counterstain.

Qualitative staining performance across experimental conditions

Photomicrographs across all 17 experimental conditions (Figures 1–17) confirmed that cashew apple aqueous extract effectively stains cell cytoplasm with a characteristic silver-like to brownish-pink hue. Primary nuclear hematoxylin staining remained well preserved across all trials, providing distinct nucleo-cytoplasmic contrast:

Effect of additives: Sections stained with ammonia-iodine modified cashew formulations (Figures 2, 3, 6, 7, 10, 11) demonstrated visibly sharper tissue outlines and higher stain intensity compared to unmordanted counterparts (Figures 4, 5, 8, 9, 12, 13).

Effect of staining duration: Extending staining exposure from 10 to 20 minutes consistently enhanced cytoplasmic colour depth and structural clarity across all unmordanted and ammonia-iodine modified extract concentrations.

Effect of glycoacetic acid: Formulations containing 0.4 mL glycoacetic acid at 1.0 g and 2.0 g concentrations

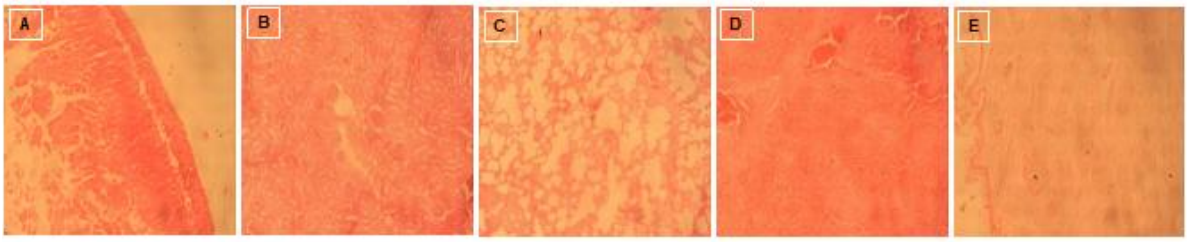


Figure 4. Photomicrographs of rat tissues counterstained with 0.3 g aqueous cashew fruit extract without additive for 10 minutes. A. Intestine (×100). B. Kidney (×400). C. Lung (×100). D. Liver (×100). E. Skin (×100)

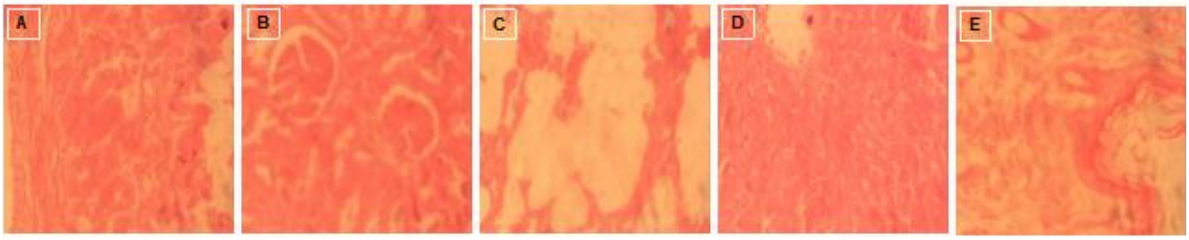


Figure 5. Photomicrographs of rat tissues counterstained with 0.3 g aqueous cashew fruit extract without additive for 20 minutes. A. Intestine (×400). B. Kidney (×100). C. Lung (×400). D. Liver (×400). E. Skin (×400).

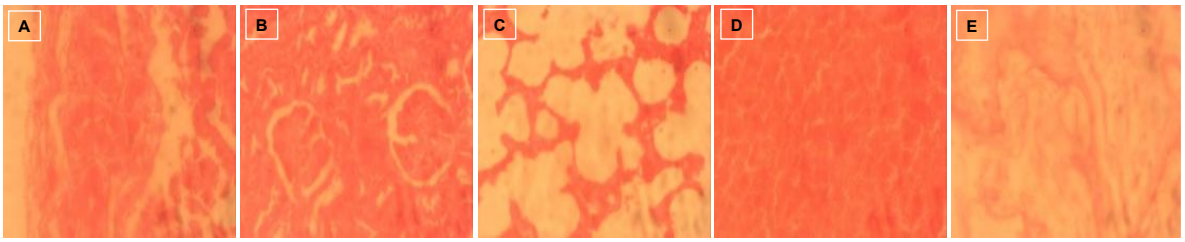


Figure 6. Photomicrographs of rat tissues counterstained with 0.15 g aqueous cashew fruit extract with ammonia-iodine additive for 10 minutes. A. Intestine (×400). B. Kidney (×400). C. Lung (×400). D. Liver (×400). E. Skin (×100).

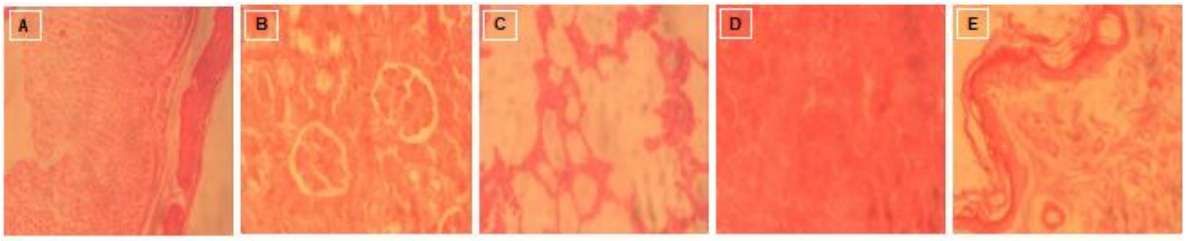


Figure 7. Photomicrographs of rat tissues counterstained with 0.15 g aqueous cashew fruit extract with ammonia-iodine additive for 20 minutes. A. Intestine (×100). B. Kidney (×400). C. Lung (×400). D. Liver (×400). E. Skin (×100).

(Figures 14–17) exhibited superior performance. Rapid counterstaining achieved within 5 to 10 minutes yielded tissue clarity and background distinction visually comparable to standard H&E controls (Figure 1).

Concentration variation: Varying extract mass from 0.15 g to 1.5 g produced minor incremental increases in colour density, with all tested concentrations successfully imparting readable cytoplasmic counterstaining.

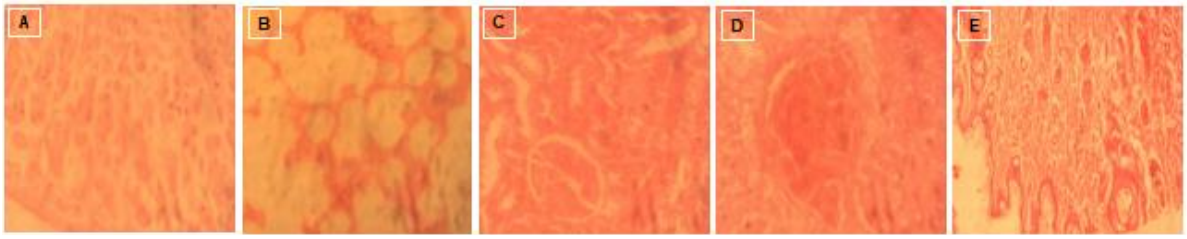


Figure 8. Photomicrographs of rat tissues counterstained with 0.15 g aqueous cashew fruit extract without additive for 10 minutes. A. Intestine (×100). B. Kidney (×400). C. Lung (×100). D. Liver (×100). E. Skin (×100).

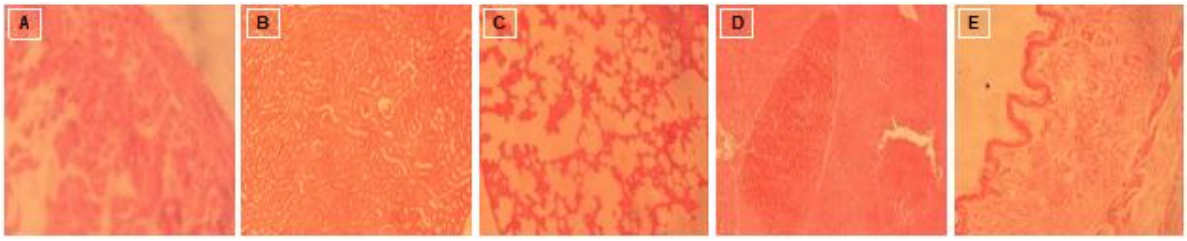


Figure 9. Photomicrographs of rat tissues counterstained with 0.15 g aqueous cashew fruit extract without additive for 20 minutes. A. Intestine (×400). B. Kidney (×100). C. Lung (×400). D. Liver (×400). E. Skin (×400).

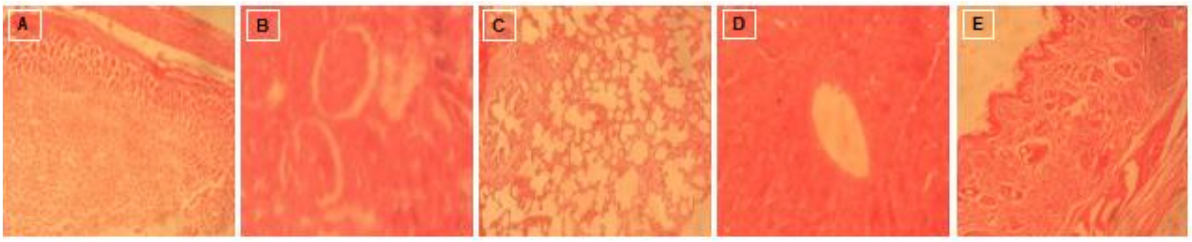


Figure 10. Photomicrographs of rat tissues counterstained with 1.5 g aqueous cashew fruit extract with ammonia-iodine additive for 10 minutes. A. Intestine (×100). B. Kidney (×400). C. Lung (×100). D. Liver (×400). E. Skin (×100).

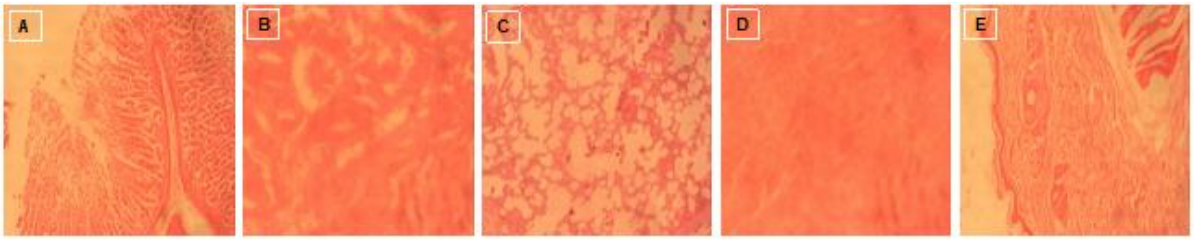


Figure 11. Photomicrographs of rat tissues counterstained with 1.5 g aqueous cashew fruit extract with ammonia-iodine additive for 20 minutes. A. Intestine (×100). B. Kidney (×400). C. Lung (×400). D. Liver (×400). E. Skin (×100).

Organ-specific staining affinity

Differential staining affinity was observed across the five harvested rat organs:

Intestine (Panel A): Mucosal epithelium and villous architecture stained vividly, providing clear morphological resolution at both ×100 and ×400 magnifications.

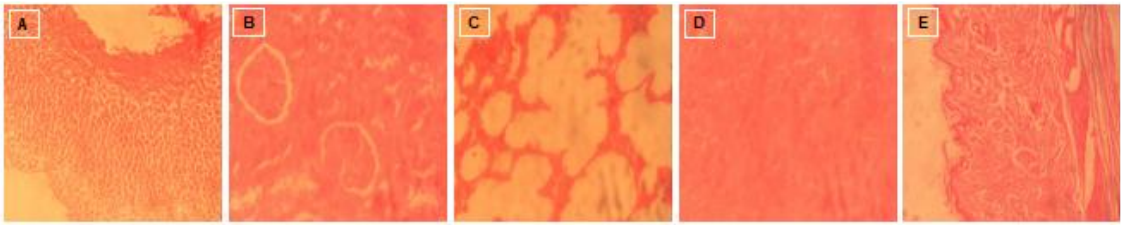


Figure 12. Photomicrographs of rat tissues counterstained with 1.5 g aqueous cashew fruit extract without additive for 10 minutes. A. Intestine (×100). B. Kidney (×400). C. Lung (×400). D. Liver (×400). E. Skin (×100).

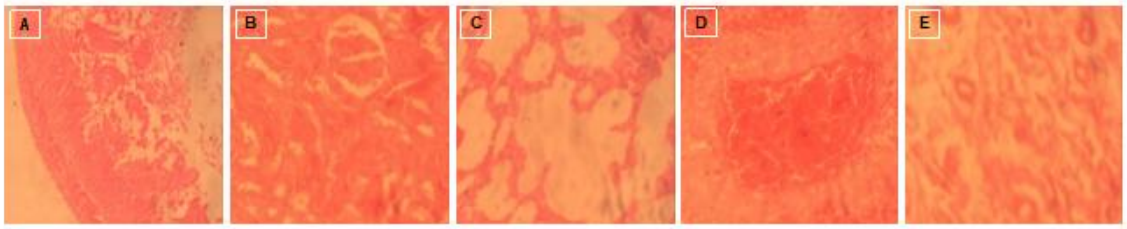


Figure 13. Photomicrographs of rat tissues counterstained with 1.5 g aqueous cashew fruit extract without additive for 20 minutes. A. Intestine (×100). B. Kidney (×400). C. Lung (×400). D. Liver (×400). E. Skin (×400).



Figure 14. Photomicrographs of rat tissues counterstained with 1.0 g aqueous cashew fruit extract with glycoacetic acid for 5 minutes. A. Intestine (×400). B. Kidney (×400). C. Lung (×100). D. Liver (×400). E. Skin (×100).



Figure 15. Photomicrographs of rat tissues counterstained with 1.0 g aqueous cashew fruit extract with glycoacetic acid for 10 minutes. A. Intestine (×400). B. Kidney (×400). C. Lung (×100). D. Liver (×400). E. Skin (×100).

Kidney (Panel B): Renal tubular epithelium and glomeruli absorbed the cashew extract with lower relative intensity compared to other organs across concentration trials; however, glycoacetic acid formulations noticeably improved renal structural definition.

Lung (Panel C): Alveolar septa and bronchial structures demonstrated high stain uptake and clean cytoplasmic differentiation.

Liver (Panel D): Hepatic cords and parenchymal lobular

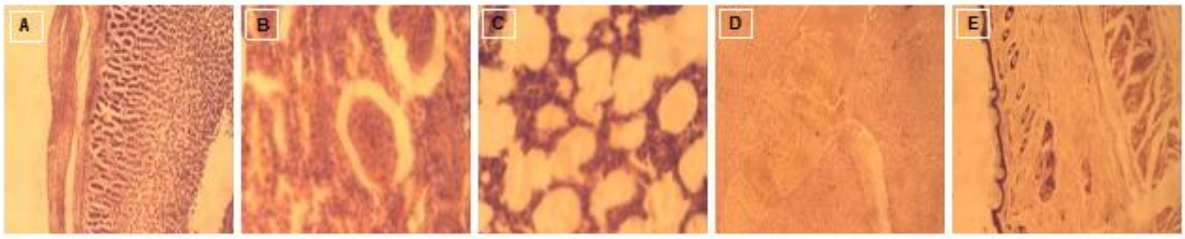


Figure 16. Photomicrographs of rat tissues counterstained with 2.0 g aqueous cashew fruit extract with glycoacetic acid for 5 minutes. A. Intestine (×100). B. Kidney (×400). C. Lung (×100). D. Liver (×100). E. Skin (×100).

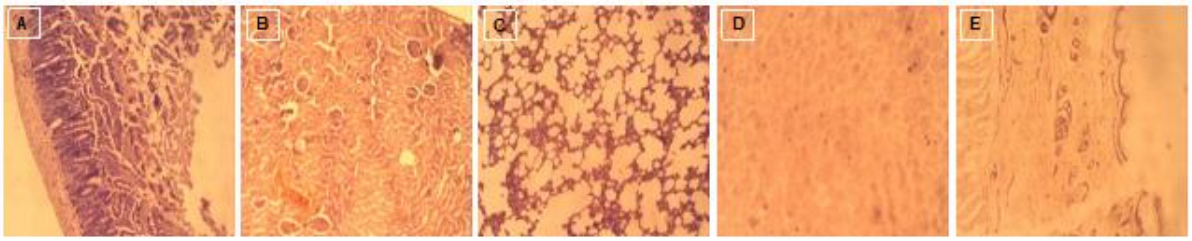


Figure 17. Photomicrographs of rat tissues counterstained with 2.0 g aqueous cashew fruit extract with glycoacetic acid for 10 minutes. A. Intestine (×100). B. Kidney (×400). C. Lung (×100). D. Liver (×100). E. Skin (×100).

layouts picked up the stain effectively, displaying clear hepatocyte cytoplasmic counterstaining.

Skin (Panel E): Epidermal layers and dermal collagen bundles stained appreciatively at ×100 magnification.

DISCUSSION

The search for sustainable, natural plant-based dyes in histopathology is driven by the imperative to reduce occupational exposure to synthetic chemical hazards and lower diagnostic laboratory expenditures. The preliminary observations of this exploratory study suggest that aqueous cashew apple (*Anacardium occidentale* L.) extract has potential utility as a natural cytoplasmic counterstain in paraffin-embedded animal tissues, providing a plant-derived alternative under selected laboratory conditions.

The proposed staining mechanism of cashew apple extract is hypothesised to be linked to its physicochemical properties, specifically its measured acidic pH of 4.6. In routine histopathology, synthetic eosin functions as an anionic acid dye that binds electrostatically to positively charged basic amino acid residues (lysine and arginine) on cytoplasmic proteins. The acidic pH of the aqueous cashew extract provides hydrogen ions that promote similar ionic binding with cytoplasmic elements, producing silver-like to brownish-pink counterstaining that contrasts clearly with hematoxylin-stained nuclei (Hartika *et al*., 2021). However, direct chemical proof of active chromophore binding mechanisms remains to be definitively established through spectroscopy.

Chemical modification played a critical role in enhancing staining quality. Incorporating an ammonia-iodine mixture improved colour density and structural sharpness (Figures 2, 3, 6, 7, 10, 11). Alkaline-iodine additives act as mordanting or trapping agents, forming insoluble complexes that facilitate dye retention during alcohol dehydration steps. Furthermore, modifying the extract with glycoacetic acid (1.0 g and 2.0 g) considerably reduced optimal counterstaining time to 5–10 minutes (Figures 14–17), aligning closely with the standard 5-minute eosin staining protocol. This rapid action is attributable to acetic acid accelerating dye penetration and inducing slight tissue swelling, which facilitates rapid dye binding.

Our observations regarding differential organ stain uptake, where intestine, lung, liver, and skin stained brightly while kidney sections exhibited lower stain absorption, reflect variations in tissue protein composition, baseline cellular metabolic machinery, and membrane density. Renal tubular epithelial cells possess dense basolateral basement membranes that may require higher extract concentrations or modified mordanting for optimal dye absorption. These findings concur with Olise *et al*. (2018), who noted that crude botanical extracts frequently exhibit organ-specific affinity variations that necessitate standardised extraction and purification.

Study Limitations: Several methodological and scientific limitations of this study should be explicitly acknowledged. First, as an exploratory pilot investigation, the sample size was limited to four albino rats (20 tissue blocks), and assessment relied entirely on qualitative visual scoring by two blinded observers rather than quantitative spectrophotometric or digital image microdensitometry analysis. Second, the active chromophoric constituents (such as specific polyphenols, flavonoids, or tannins) in the crude cashew extract were not isolated or quantified. Third, this study did not perform formal acute, subacute, or chronic cellular toxicity assays, nor did it evaluate the economic cost-benefit ratio or batch-to-batch chemical stability. Finally, long-term light-fastness and antifungal preservative stability of the reconstituted staining solutions were not investigated in this initial phase.

Potential Clinical and Practical Implications: Despite these limitations, the demonstration that cashew apple extract, an abundant agricultural waste product across tropical regions, can impart readable cytoplasmic counterstaining holds potential clinical and educational value. In resource-limited diagnostic and academic laboratories, validated botanical stains could serve as eco-friendly auxiliary reagents, reducing reliance on commercial synthetic dyes once standardised extraction, safety, and stability protocols are established (Barde *et al*., 2021; Melford, 2025).

Conclusion

This exploratory study demonstrated that aqueous cashew apple (*Anacardium occidentale* L.) extract, particularly when modified with glycoacetic acid, imparts visible cytoplasmic counterstaining in paraffin-embedded rat tissues with visual clarity comparable to standard synthetic eosin. The measured acidic pH (4.6) of the extract facilitates cytoplasmic dye uptake while preserving nuclear hematoxylin contrast. However, formal claims regarding non-toxicity, cost-effectiveness, or quantitative technical equivalence to commercial eosin cannot be made based on these preliminary visual data.

Recommendations

Based on these empirical findings, it is recommended that further studies systematically evaluate the shelf-life, antifungal preservation, light-fastness, and batch-to-batch reproducibility of reconstituted staining solutions over extended storage periods to ensure consistent and effective tissue staining.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ACKNOWLEDGEMENTS

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