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From Nature to Microscopy: Plant-Based Alternatives to Hematoxylin and Eosin in Oral Histopathology

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Abstract

Hematoxylin and eosin (H&E) remains the reference stain for routine histopathology because it consistently differentiates nuclei, cytoplasm, and extracellular matrix. Interest in plant-derived stains has increased because of reagent access, cost, occupational exposure, and environmental considerations. This narrative review evaluates turmeric, beetroot, pomegranate, blueberry, kumkum, ginger, henna, hibiscus, and related botanical materials as histological colorants, with emphasis on oral tissues. Published studies demonstrate tissue affinity for curcuminoids, betalains, anthocyanins, lawsone, and other plant pigments, but performance varies substantially with botanical source, extraction chemistry, pH, mordant, staining time, tissue processing, and storage. Illustrative observations from a student teaching laboratory exercise using author-provided cheek-swab buccal cells are described separately from published histopathological evidence. The recorded classroom scores favored H&E over the botanical preparations, but the exercise was not designed to establish diagnostic performance, tissue selectivity, or environmental benefit. Current evidence supports further formulation development and carefully bounded educational or adjunctive use rather than replacement of H&E in diagnostic practice. Progress requires chemically characterized preparations, locked protocols, blinded multiobserver evaluation, objective image analysis, stability studies, and diagnostic-performance endpoints. This narrative review aims to evaluate plant-based alternatives to H&E for oral histopathology, focusing on pigment chemistry, proposed tissue-binding mechanisms, reported staining performance, and limitations requiring further validation.

Categories: Dentistry
Keywords: anthocyanins, betalains, curcumin, hematoxylin and eosin, histological staining, natural dyes, oral pathology

Introduction And Background

Histopathological interpretation depends on reproducible optical contrast and preservation of tissue morphology. Routine hematoxylin and eosin (H&E) staining provides complementary nuclear and cytoplasmic contrast: oxidized hematoxylin, usually complexed with a metal mordant, preferentially demonstrates basophilic structures, while eosin colors protein-rich cytoplasmic and extracellular components in pink-to-red shades [1]. Its established role reflects standardized processing, familiar color conventions, quality-control systems, and decades of diagnostic training [1,2]. Hematoxylin is itself derived from logwood (*Haematoxylum campechianum*); consequently, investigating plant-derived alternatives does not necessarily involve replacing both components. Many studies evaluate substitutes for eosin while retaining hematoxylin for nuclear staining.

Interest in plant-derived stains reflects potential advantages in local availability, renewable sourcing, and reagent access [2]. However, natural origin does not establish safety, sustainability, or diagnostic suitability. Plant extracts can vary in composition, contain contaminants or sensitizers, and remain part of workflows involving ethanol, acids, xylene, and synthetic mountants [2,3]. Candidate preparations therefore require evaluation as complete reagent systems, including preparation, processing, handling, and storage.

Here, “plant-derived stain” denotes a staining preparation obtained from plant material, including crude extracts, isolated pigments, and formulated reagents containing these pigments. “Botanical stain” is synonymous with this term, although “plant-derived stain” is used consistently throughout. “Natural stain” encompasses nonplant sources as well, whereas “alternative dye” denotes a proposed substitute for a conventional dye regardless of origin. Specific pigments and preparations are identified by name wherever possible.

Published studies establish that selected curcuminoid, betalain, anthocyanin, and lawsone-containing preparations can produce histological coloration, although results depend on extraction, formulation, staining conditions, and tissue processing [3-8]. What remains uncertain is whether these preparations provide reproducible selectivity, adequate storage stability, and diagnostic performance comparable to

conventional staining. Proposed pigment-tissue interactions offer a chemical rationale for staining but do not independently establish performance in fixed, processed tissue [9-11]. Strong coloration alone is insufficient: evaluation must also address nuclear detail, cytoplasmic differentiation, background staining, and preservation of morphological boundaries [1,3,8].

A focused assessment of oral histopathology is needed because candidate stains must preserve distinctions among epithelial layers, keratinized surfaces, connective tissue, and inflammatory components. Assessment of epithelial dysplasia, abnormal epithelial maturation accompanied by cellular and architectural atypia, requires clear nuclear detail and preservation of epithelial organization. Recognition of invasion requires visualization of tumor cells extending into the underlying connective tissue. Candidate stains must therefore preserve these diagnostic features rather than merely produce visible coloration. Findings from other tissue types or isolated pigment-binding experiments cannot alone establish suitability for interpreting oral lesions. The relevant question is whether a preparation consistently reveals the morphological features needed for oral histopathological assessment.

The aim is to critically synthesize evidence addressing four questions: which plant-derived preparations have been investigated; how pigment chemistry and formulation relate to reported staining performance; what evidence supports their application to oral tissues; and which methodological gaps and validation requirements remain before clinical adoption. Emphasis is placed on distinguishing plausible mechanisms from demonstrated staining performance and diagnostic utility.

Review

Why H&E remains the reference method

Hematoxylin is oxidized to hematein and combined with a mordant to create a dye lake that demonstrates chromatin and other basophilic structures. Eosin is an anionic xanthene dye that interacts with cationic groups in cytoplasmic and extracellular proteins [1]. Diagnostic value arises from selective affinity, crisp boundaries, low background, balanced nuclear-to-cytoplasmic contrast, compatibility with formalin-fixed paraffin-embedded tissue, and durable mounted slides. A substitute that merely colors tissue is therefore insufficient; it must preserve the morphologic features needed to recognize dysplasia, invasion, inflammation, keratinization, salivary differentiation, vascular structures, and tumor-stroma relationships.

Published botanical-stain studies often assess intensity or attractiveness but do not measure whether pathologists reach the same diagnosis, grade, or margin determination as with H&E [2,3,8]. Replacement claims consequently require paired diagnostic studies with predefined acceptance criteria, whereas exploratory coloration studies should be described as feasibility evidence.

The H&E reaction is itself sensitive to laboratory conditions. Hematoxylin performance depends on oxidation state, mordant species, formulation age, differentiation, and bluing, whereas eosin intensity depends strongly on pH, concentration, alcohol carryover, and staining time [1]. These variables are controlled in routine laboratories through standardized operating procedures, control tissues, reagent-change schedules, and review of staining trends. Botanical alternatives must be held to the same quality principles; comparison with an inadequately controlled H&E run would not provide a valid benchmark. Table *1* summarizes the principal pigment classes, their extraction and staining characteristics, reported tissue targets, and limitations.

Pigment class and principal molecules	Representative sources	Extraction and staining behavior	Reported tissue targets	Key limitations and citations	References
Curcuminoids: curcumin, demethoxycurcumin, bisdemethoxycurcumin	Curcuma longa; some traditional kumkum preparations	Poor water solubility; commonly extracted in ethanol. Protein association may involve hydrophobic contacts and hydrogen bonding; pH also affects ionization and stability.	Cytoplasm, collagen, muscle, red blood cells, connective-tissue elements	Yellow background, fading, solvent dependence, and variable pigment content.	[3-5,9,12]
Betalains: betanin, betanidin, vulgaxanthins	Beta vulgaris beetroot	Water-soluble red-violet betacyanins and yellow betaxanthins. Yield and spectral profile differ between aqueous and ethanolic extracts.	Muscle, mucin, keratin, nerve fibers, red blood cells; variable nuclear coloration	Sensitive to heat, oxygen, light, pH, and prolonged processing.	[6,8]
Anthocyanins: cyanidin, delphinidin, pelargonidin glycosides	Pomegranate, blueberry, hibiscus, rose, red cabbage	Water-soluble flavonoids interconvert among colored molecular forms as pH changes. Acidification and refrigeration may improve color expression or storage.	Epithelium, basement membrane, inflammatory cells, collagen, and other stromal elements	Strong pH dependence, source variability, oxidation, fading, and possible nonspecific background.	[7,8]
Naphthoquinones and related phenolics: lawsone	Lawsonia inermis henna	Lawsone-containing extracts can produce orange to red-brown coloration. Protein association is plausible, but the dominant mechanism in fixed tissue remains unconfirmed.	Cytoplasm, collagen, salivary structures, bone, and other acellular components	Composition varies by source and commercial processing; color differs from conventional eosin.	[8,13]
Mixed or incompletely defined pigments	Kumkum, ginger, onion skin, saffron-containing products	Behavior depends on formulation. Commercial kumkum may contain turmeric, lime, mineral salts, saffron, or synthetic colorants.	Cytoplasm, epithelium, muscle, vessels, nerve bundles, and red blood cells in selected reports	Identity, additives, contamination, batch consistency, and external reproducibility are often inadequately documented.	[3,8,14-16]

TABLE 1: Chemical and methodological characteristics of botanical pigment groups

In practical terms, a botanical extract is usually a multicomponent chemical system rather than a single dye. Co-extracted acids, sugars, tannins, proteins, minerals, and aromatic compounds may shift pH, stabilize pigment through copigmentation, compete for binding sites, or generate background. Metal ions may create new chromophores through coordination complexes; Al^{3+}, for example, can convert selected roselle anthocyanins into violet-blue nuclear-selective complexes without prior oxidation [10]. Consequently, formulation science must distinguish the coloring molecule, its stabilizing matrix, the mordant or complexing agent, and the final working-solution environment. Figure *1* provides a conceptual overview of their color and stability behavior.

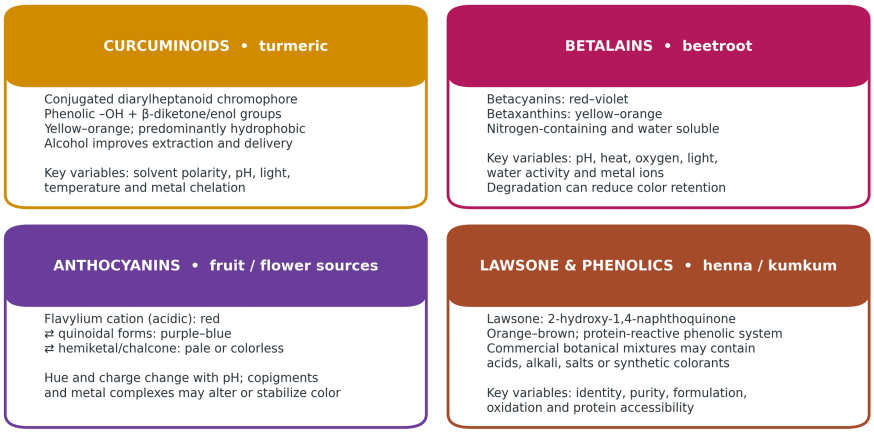
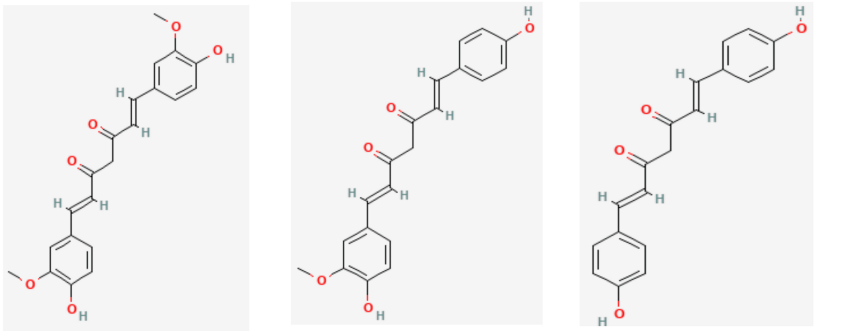


FIGURE 1: Botanical pigment classes and formulation variables

This conceptual schematic was created using ChatGPT (OpenAI, San Francisco, CA, USA), a generative artificial intelligence (AI) tool to illustrate the relationships discussed in the review. It does not represent experimental measurements or microscopy data. Conceptual overview of the principal botanical pigment classes and formulation variables relevant to histological staining. Curcuminoids, betalains, anthocyanins, and lawsone-containing or mixed phenolic preparations differ in solubility, ionization, color, and stability. Lawsone is associated with henna; kumkum is a multicomponent preparation and should not be assumed to contain lawsone. The diagram summarizes proposed relationships and does not establish tissue selectivity for a particular formulation. The chemical principles are discussed in the cited literature [9-12].

Turmeric and curcuminoid stains

Turmeric rhizomes contain a family of conjugated diarylheptanoids rather than a single dye. The principal chromophores are curcumin, demethoxycurcumin, and bisdemethoxycurcumin; their relative abundance varies with cultivar, geography, processing, and extraction. Each contains two aromatic rings connected by a conjugated heptadienedione chain, but progressive removal of aromatic methoxy groups changes electron donation, polarity, spectral behavior, and degradation susceptibility [12,17,18]. All three are sparingly soluble in water and are commonly extracted into ethanol or another organic-rich phase. Consequently, a turmeric powder extract, an analytically defined three-curcuminoid mixture, and purified curcumin should be treated as different candidate stains. Figure *2* compares the chemical structures of the three principal curcuminoids and highlights their differing methoxy-substitution patterns.



Curcumin $C_{21}H_{20}O_6$ | Demethoxycurcumin $C_{20}H_{18}O_5$ | Bisdemethoxycurcumin $C_{19}H_{16}O_4$

FIGURE 2: Curcumin and its principal demethoxy derivatives

Chemical structure images were downloaded from the corresponding PubChem compound records and assembled into a panel in Microsoft PowerPoint (Microsoft Corp., Redmond, WA, USA), then exported as a JPG for submission. The chemical structure depictions were obtained from PubChem and were not generated using AI. Chemical structures of curcumin (PubChem CID 969516), demethoxycurcumin (PubChem CID 5469424), and bisdemethoxycurcumin (PubChem CID 5315472) in the forms depicted in the downloaded PubChem images. The compounds contain two, one, and no aromatic methoxy groups, respectively. All three compounds retain the conjugated curcuminoid backbone. Keto-enol tautomerism, ionization, and metal coordination depend on solvent, pH, and formulation. Compound identities are referenced to PubChem records; the PubChem database is described by Kim et al. [19]. Curcuminoid chemistry and stability are described separately [12,17,20].

CID, compound identifier

Curcumin

Curcumin, chemically diferuloylmethane, bears two phenolic hydroxyl groups, two aromatic methoxy groups, and a central beta-diketone system. It undergoes keto-enol tautomerism; in many nonpolar or moderately polar environments, the conjugated enol is favored and sustains the extended chromophore responsible for intense yellow color. The beta-diketone/enolate region can coordinate metal ions, while the phenolic and carbonyl groups can act as hydrogen-bond donors or acceptors. Curcumin is lipophilic, poorly water-soluble, and readily affected by solvent polarity, pH, light, oxygen, and temperature. In neutral-to-alkaline aqueous media, it undergoes hydrolytic and oxidative transformation, whereas hydrophobic microenvironments and organic solvents can increase apparent persistence [9,12,21].

In formalin-fixed paraffin-embedded tissue, curcumin retention is most plausibly noncovalent. Its aromatic and conjugated surfaces may associate with hydrophobic protein domains and aromatic side chains; phenolic and carbonyl groups may form hydrogen bonds with peptide backbones and polar amino acid residues; and, when sufficiently ionized, phenolate or enolate species may participate in electrostatic interactions. Spectroscopic studies confirm binding of curcumin and turmeric extracts to serum albumin, including retention near hydrophobic protein sites, but they do not establish the dominant mechanism in fixed tissue [22,23]. Thus, proposed hydrophobic, hydrogen-bonding, and pi-associated interactions should be regarded as mechanistic hypotheses for histology rather than demonstrated covalent staining reactions.

Purified curcumin is the most experimentally tractable curcuminoid stain because concentration and purity can be specified. However, its instability creates a narrow formulation window: raising pH may increase ionization and apparent tissue interaction while simultaneously accelerating decomposition; excessive aqueous carryover can precipitate or extract the pigment; and prolonged illumination can bleach the working solution. A development protocol should therefore specify curcumin concentration, ethanol fraction, final pH, absorbance maximum and peak area, staining time, water content, light protection, and maximum solution age [9,17,21].

Demethoxycurcumin

Demethoxycurcumin contains one aromatic methoxy group rather than the two present in curcumin. It retains the conjugated beta-diketone chromophore and both phenolic hydroxyl groups, so keto-enol tautomerism, hydrogen bonding, hydrophobic association, and metal coordination remain chemically plausible. Loss of one methoxy substituent reduces electron donation and slightly alters polarity and electronic distribution; therefore, demethoxycurcumin should not be assumed to have the same molar

absorptivity, color yield, tissue partitioning, or wash resistance as curcumin [12,17,18].

Direct histological studies using isolated demethoxycurcumin are scarce. On chemical grounds, its tissue binding is expected to involve adsorption to protein-rich cytoplasm and extracellular matrix through a combination of hydrophobic contacts and hydrogen bonds, with a possible ionic contribution after phenolic or enolic deprotonation. Comparative stress testing found demethoxycurcumin more resistant than curcumin but less resistant than bisdemethoxycurcumin under acidic, alkaline, and oxidative conditions [17]. This intermediate stability may be advantageous in an alcoholic counterstain, but it requires confirmation by side-by-side staining, accelerated aging, and post-mounting color-retention studies rather than inference from solution chemistry alone.

Bisdemethoxycurcumin

Bisdemethoxycurcumin lacks both aromatic methoxy groups and retains two phenolic hydroxyls plus the beta-diketone bridge. The lower methoxylation changes resonance donation, lipophilicity, and steric environment while preserving the extended conjugated system required for yellow coloration. Under several forced-degradation conditions, the reported stability order was bisdemethoxycurcumin greater than demethoxycurcumin greater than curcumin; all three nevertheless showed pronounced sunlight sensitivity [17]. The comparatively greater chemical stability makes bisdemethoxycurcumin a rational candidate for a longer-lived working stain, although its extraction yield and optical intensity may differ from those of curcumin.

Its proposed fixed-tissue binding remains primarily noncovalent: phenolic and diketone groups permit hydrogen bonding and ionization-dependent interactions, whereas the conjugated aromatic scaffold can partition into hydrophobic protein regions. Removal of methoxy groups may reduce hydrophobic contact at some sites while increasing the relative contribution of phenolic interactions. These competing effects make tissue affinity impossible to predict reliably from structure alone. Purified bisdemethoxycurcumin should therefore be compared at matched molar concentration and matched absorbance, with identical differentiation, dehydration, clearing, and mounting steps.

Defined curcuminoid mixtures and crude turmeric extracts

Most published histological turmeric preparations are multicomponent extracts rather than isolated stains. High-performance liquid chromatography (HPLC) can resolve and quantify curcumin, demethoxycurcumin, and bisdemethoxycurcumin and should be used to define mixture composition when reproducibility is the goal [18]. Mixtures may behave differently from isolated constituents: Peram et al. found lower degradation in mixed curcuminoids than in the corresponding pure compounds under several stress conditions, suggesting matrix-dependent stabilization [17]. Conversely, crude rhizome extracts also contain essential oils, polysaccharides, proteins, minerals, and other phenolics that may change pH, solubility, background deposition, and tissue wetting.

Avwiorio et al. reported staining of collagen fibers and red blood cells with fractionated Curcuma longa extract, with 70% ethanol and approximately neutral-to-acidic formulations outperforming several alternative solvent and mordant conditions [4]. Kumar et al. likewise demonstrated tissue staining and emphasized concentration and exposure time [5]. In oral tissues, Sudhakaran et al. used an alcoholic extract for approximately nine minutes after hematoxylin; epithelium and connective tissue became yellow to golden yellow, but background, overall viewing quality, and fading were less favorable than with ginger [3]. Deepthi et al. later found moderate turmeric performance in a six-candidate comparison, although its composite score remained below eosin [8]. These results establish staining potential but not diagnostic equivalence or lot-to-lot reproducibility. Curcumin has also been evaluated specifically as a substitute for eosin in routine histological staining [20].

A defensible mixed-curcuminoid reagent should report botanical source and voucher information, extraction mass-to-volume ratio, solvent composition, extraction temperature and duration, filtration, dry-residue yield, final pH, visible absorbance, and HPLC percentage of each principal curcuminoid. Acceptance limits should include particulate burden, short-term photostability, staining intensity, background, and color retention after dehydration and mounting. Reporting only “turmeric extract” prevents meaningful replication because two extracts can have similar visual color but substantially different chromophore ratios and nonpigment matrices.

Curcuminoid-metal complexes and mordanted formulations

Curcuminoids are chelating ligands. Deprotonation of the beta-diketone enol allows coordination through oxygen atoms, and phenolic oxygen may also contribute depending on the metal, pH, and stoichiometry. Complexation with aluminum, iron, copper, zinc, boron, and other ions can alter electron delocalization, shift absorption, change solubility, and modify net charge [24]. In principle, a metal ion can act as a mordant by forming a colored curcuminoid complex with increased tissue retention or by bridging ligand and tissue functional groups. In practice, the coordination species present in an alcoholic histological bath may be heterogeneous and strongly pH-dependent.

Metal-assisted formulations therefore require separate toxicological and analytical treatment from uncomplexed turmeric. The metal identity, oxidation state, ligand-to-metal ratio, counterion, pH, preparation order, aging time, and spectral maximum should be defined. Precipitation, metachromatic color shifts, nonspecific background, and interference with hematoxylin must be assessed. Because the histological literature has not yet established which coordination structure binds which fixed-tissue target, claims of "mordant binding" should remain provisional until supported by competition experiments, elemental mapping, spectroscopy, or controlled comparisons of ligand alone, metal alone, and the preformed complex [24].

Taken together, the curcuminoids constitute a related but noninterchangeable set of candidate stains. Curcumin offers the largest evidence base, demethoxycurcumin provides an intermediate structural and stability profile, and bisdemethoxycurcumin offers comparatively greater stress stability. Future comparisons should use purified compounds and a composition-defined mixture in parallel, normalize dose by molarity or absorbance, and quantify tissue-specific optical density, contrast, background, interobserver agreement, and post-mounting fading. Such experiments would reveal whether performance arises from one dominant chromophore, cooperative mixture effects, or co-extracted turmeric constituents.

Beetroot and betalain stains

Beetroot contains red-violet betacyanins, predominantly betanin, and yellow betaxanthins. Unlike anthocyanins, betalains are nitrogen-containing pigments and generally occur in different botanical families. Udonkang et al. compared aqueous and ethanolic beetroot extracts using spectrophotometry and albino rat tissues. The extracts showed different absorption characteristics and stained muscle, mucin, red blood cells, keratin, and nerve fibers, demonstrating that extraction chemistry changes both pigment yield and histological behavior [6].

Betalain performance is constrained by chemical instability. Temperature, oxygen exposure, light, water activity, pH, metal ions, and storage time can accelerate degradation or shift visible color. Histological protocols have therefore used fresh extracts, acidification, vinegar rinses, refrigeration, or shortened processing, but these modifications are not standardized. Excessive acidity may also alter tissue appearance or diminish hematoxylin, while dehydration and clearing can extract or attenuate water-soluble pigment.

The published results are mixed. Beetroot has produced recognizable coloration in animal tissues and cytological or microbiological preparations, but it has also shown low contrast and poor nuclear and cytoplasmic detail in comparative oral-tissue studies [6,8]. Deepthi et al. used an 80% ethanolic extract with vinegar rinses and a 30-60-minute staining interval; beetroot ranked among the lowest-performing candidates relative to eosin [8]. Obeta et al. likewise concluded that beetroot and turmeric showed individual staining potential but that their combined use did not reproduce routine H&E quality [25]. These findings suggest that betalain concentration and preservation must be quantified before performance can be meaningfully compared across laboratories.

Betanin and isobetanin absorb in the green region and therefore appear red-violet, while betaxanthins absorb at shorter wavelengths and contribute yellow-orange tones. Hydrolysis, oxidation, decarboxylation, dehydrogenation, and isomerization can change both absorbance and tissue color. Controlled studies show that individual betalains have different pH-response and storage profiles; prolonged strongly alkaline exposure causes major degradation, whereas several principal pigments are relatively more stable under acidic conditions [11]. A beetroot stain should therefore be treated as a defined ratio of multiple chromophores, not as a single red dye. HPLC or liquid chromatography-mass spectrometry (LC-MS) profiling is ideal, but reproducible absorbance ratios and pH specifications may be feasible for routine development laboratories.

Pomegranate and other anthocyanin sources

Anthocyanins are water-soluble glycosides of anthocyanidins that generate red, purple, and blue coloration in many fruits, flowers, and pigmented grains. The B-ring hydroxylation pattern distinguishes common aglycones such as cyanidin, delphinidin, pelargonidin, petunidin, peonidin, and malvidin; glycosylation increases aqueous solubility, whereas acylation and copigmentation can improve color stability. Anthocyanins occupy a pH-dependent reaction network: strongly acidic conditions favor the red flavylium cation, increasing pH promotes purple or blue quinoidal forms, and hydration can generate pale hemiketals and chalcones. Light, oxygen, temperature, enzymes, metals, and solvent composition further influence degradation and visible absorbance [10,26-32].

In fixed tissue, the most plausible retention mechanisms are electrostatic attraction between charged anthocyanin species and oppositely charged tissue groups, hydrogen bonding through phenolic hydroxyl and glycosidic groups, hydrophobic and pi-associated interactions between aromatic rings and protein side chains, metal-ion coordination, and physical entrapment within cross-linked macromolecular networks. Spectroscopic studies of anthocyanin-protein complexes support contributions from hydrogen bonding, hydrophobic forces, and electrostatic interactions [26]. These observations establish chemical plausibility but do not prove which mechanism dominates in formalin-fixed paraffin-embedded sections. Each source

must therefore be evaluated as a specific formulation rather than as a generic anthocyanin stain.

Pomegranate

Pomegranate arils contain cyanidin-, delphinidin-, and pelargonidin-based mono- and diglucosides, whereas peel preparations are commonly richer in hydrolyzable tannins, particularly ellagitannin-related compounds, and other phenolics. Anthocyanin profiles vary with cultivar, maturity, climate, plant part, processing, and storage; cold storage and pasteurization can change both total concentration and the relative abundance of individual pigments [27,33,34]. Pomegranate extract is therefore not a chemically singular reagent, and aril juice should not be treated as equivalent to peel decoction.

Sachdev et al. applied pomegranate-derived anthocyanin preparations to formalin-fixed oral tissues and observed magenta coloration in basal and suprabasal epithelial cells, basement membrane, inflammatory cells, and collagen [7]. The acidic flavylium form may contribute ionic attraction to basic protein sites, while hydroxyl-rich anthocyanins and co-extracted tannins can form multiple hydrogen bonds with collagen and cytoplasmic proteins. Aromatic stacking and nonspecific adsorption may further increase retention; tannins may intensify protein association but can also increase background. Lemon extract, refrigeration, 4% acetic acid, slide orientation, and solution age affected the reported protocol, emphasizing that acidity and reagent handling are active experimental variables [7].

The classroom pomegranate observations described below concern cheek-swab buccal cells and cannot establish staining performance in tissue sections. In future tissue-staining studies, weak contrast could reflect low anthocyanin concentration, conversion to hemiketal or chalcone forms, insufficient mordanting, extraction during dehydration, or competition from peel tannins and other matrix components. A reproducible preparation should specify cultivar, plant part, extraction solvent and ratio, final pH, total monomeric anthocyanin concentration, chromatographic markers, refrigerated shelf life, and color retention after alcohol, clearing, and mounting [7,27,33,34].

Blueberry

Blueberries contain a chemically diverse anthocyanin mixture derived mainly from malvidin, delphinidin, petunidin, cyanidin, and peonidin, commonly glycosylated with glucose, galactose, or arabinose. This diversity broadens visible color but creates differential stability among components. Liu et al. identified ten blueberry anthocyanins and showed that low temperature and low pH improved total and individual pigment stability, whereas strongly alkaline conditions and elevated temperature accelerated loss [28].

Blueberry anthocyanins are expected to bind tissue through the general noncovalent mechanisms described above, but the balance changes with pH because molecular charge and hydration state change simultaneously. Delphinidin-rich species offer more hydroxyl groups for hydrogen bonding and metal coordination, whereas methoxylated malvidin derivatives may be relatively more hydrophobic. A blueberry-kumkum mixture is particularly difficult to interpret because alkaline kumkum can shift anthocyanins away from the red flavylium state and accelerate degradation. Blueberry should therefore be optimized alone before combination studies, using acidified, light-protected extracts and defined storage limits [26,28].

Hibiscus and roselle

Hibiscus rosa-sinensis and *Hibiscus sabdariffa* contain anthocyanins, flavonols, phenolic acids, and tannins, although their pigment profiles differ by species, cultivar, and plant part. Roselle calyces commonly contain delphinidin- and cyanidin-based glycosides, while ornamental hibiscus preparations may have a different and less well-defined profile. Acidic flavylium species may interact electrostatically with basic tissue groups, and their hydroxyl groups support hydrogen bonding; aromatic rings permit hydrophobic or pi-associated contacts. Co-extracted tannins can strengthen protein association but may also increase precipitate and background [10,26,35].

Crude hibiscus preparations have produced eosin-like cytoplasmic coloration in selected studies, but performance was poor in a recent comparative oral-tissue evaluation [8,35,36]. By contrast, Alshamar and Dapson used controlled aluminum complexation to produce a violet-blue, nuclear-selective roselle stain without prior oxidation [10]. Aluminum coordination changes the anthocyanin chromophore and may create a positively charged or amphoteric dye complex with different tissue affinity from the crude extract. A roselle-aluminum complex and a simple hibiscus decoction are therefore distinct reagents and should not be pooled analytically.

Rose petals

Rose-petal color commonly arises from cyanidin glycosides, accompanied by flavonols and other phenolics; composition and concentration vary greatly among cultivars. Chemical profiling of multiple rose cultivars has confirmed marked variation in total anthocyanins and the presence of several individual anthocyanin compounds [32]. For histological development, cultivar selection is therefore a formulation variable, not merely a botanical descriptor.

Cyanidin glycosides can form hydrogen bonds with proteins and polysaccharides, participate in aromatic interactions, and display pH-dependent electrostatic behavior. Flavonols can act as copigments through pi-stacking, shifting absorbance and partially shielding the chromophore from hydration. However, rose-petal staining evidence remains preliminary, and fragrance oils, sugars, tannins, and extraction debris may contribute nonspecific background. Candidate preparations should be fractionated and compared with purified cyanidin-3-glucoside controls before tissue selectivity is attributed to rose anthocyanins [26,32].

Black rice

The pigmented pericarp of black rice is dominated by cyanidin-3-glucoside, with peonidin-3-glucoside and other minor anthocyanins reported in different varieties. Pedro et al. identified cyanidin-3-glucoside as the principal anthocyanin and showed that extraction temperature, time, solid-to-solvent ratio, pH, light, and copigments changed yield and stability [29]. The starch, protein, lipid, and phenolic matrix of rice bran can also influence extraction and filtration.

Cyanidin-3-glucoside provides a flavylium chromophore for acidic staining, phenolic hydroxyl groups for hydrogen bonding, and an aromatic surface for hydrophobic or pi interactions. Gallic acid, phytic acid, and other co-extracted compounds can alter copigmentation or metal availability, thereby modifying hue and binding indirectly [29]. Because black-rice extracts are dark and chemically dense, excessive concentration may create precipitate or mask morphology. Clarification, marker quantification, and controlled differentiation are essential before comparing performance with H&E.

Mulberry

Mulberry fruit pigment is composed mainly of cyanidin-3-O-rutinoside and cyanidin-3-O-glucoside, with smaller pelargonidin derivatives. Qin et al. reported approximate relative abundances of 60% cyanidin-3-O-rutinoside and 38% cyanidin-3-O-glucoside in a characterized extract [30]. The rutinoside group increases molecular size and hydrophilicity relative to the glucoside, which may slow diffusion into fixed tissue while increasing opportunities for hydrogen bonding.

Mulberry anthocyanins may associate with tissue proteins through electrostatic, hydrogen-bonding, and hydrophobic interactions, but glycoside size, aggregation, and co-extracted sugars can influence penetration and washout. Acidified ethanol can improve extraction and maintain red flavylium species, yet subsequent dehydration may remove weakly retained pigment. Histological studies should compare purified cyanidin glucoside and rutinoside fractions to determine whether molecular size improves selectivity or merely reduces penetration [26,30].

Red cabbage

Red cabbage contains structurally complex cyanidin glycosides that are frequently mono- or diacylated with hydroxycinnamic acids. Intramolecular pi-stacking between acyl groups and the cyanidin chromophore protects against hydration and can stabilize purple-blue color more effectively than nonacylated anthocyanins. Fenger et al. showed that acylation, iron binding, and antioxidant environment materially affected color loss and degradation in neutral solution [31].

Acylated structures may offer useful resistance to aqueous fading and additional aromatic surfaces for hydrophobic association with tissue proteins. Metal coordination can shift hue and increase stability, while multiple sugar and hydroxyl groups support hydrogen bonding. The same molecular size and charge that improve stability may slow tissue diffusion, however, and iron or aluminum complexes may change selectivity. Red-cabbage formulations should therefore be developed as defined anthocyanin-metal systems with metal-only controls rather than as crude juice stains [10,26,31].

Across all anthocyanin sources, future studies should report botanical identity, cultivar, plant part, extraction conditions, final pH and buffer capacity, total monomeric anthocyanin content, chromatographic fingerprint, mordant concentration, absorbance maximum, storage conditions, and pigment retention after dehydration and mounting. Single-source extracts, purified-marker controls, solvent controls, and mordant-only controls are necessary to distinguish tissue binding from transient coloration, precipitate, or metal deposition [10,26-32].

Kumkum, henna, ginger, and other botanical candidates

This group should not be treated as chemically uniform. Kumkum is a manufactured preparation, whereas henna and ginger are botanical extracts containing multiple chromophores and nonchromophoric constituents. Other candidates, including onion skin, sorghum, saffron, annatto, and paprika, represent different pigment families with different ionization, solubility, diffusion, and stability profiles. The following subsections therefore distinguish observed histological performance from proposed molecular mechanisms; direct binding studies in formalin-fixed paraffin-embedded tissue remain limited.

Kumkum

Kumkum is a cultural and manufactured colorant rather than a single defined chemical substance. A traditional herbal preparation may be made by combining turmeric or *Curcuma aromatica* with slaked lime, whereas commercial powders may additionally contain calcium carbonate, mineral pigments, saffron, fragrances, preservatives, or synthetic dyes. Lavanya et al. evaluated a deliberately prepared *Curcuma aromatica*-slaked lime formulation and reported useful differential staining of oral tissues [14]. Kumkum has also been investigated as a counterstain in cervical and routine histological sections, with reported coloration of epithelium, connective tissue, blood vessels, smooth muscle, nerve bundles, and erythrocytes [15,16]. In a comparative oral-tissue study, kumkum performed below eosin and henna but above several other botanical candidates [8].

Its color chemistry depends strongly on formulation. Curcuminoids contain phenolic groups and a conjugated beta-diketone system. Addition of calcium hydroxide raises pH, promotes deprotonation of phenolic or enolic groups, and shifts the electron distribution of the chromophore, producing the characteristic red-to-brown alkaline color. Calcium ions may also associate with oxygen-donor sites, but a reproducible curcuminoid-calcium dye lake in fixed tissue has not been conclusively demonstrated. The most defensible proposed binding mechanisms are hydrogen bonding through phenolic and carbonyl groups, hydrophobic association of the aromatic conjugated scaffold with protein-rich tissue, nonspecific adsorption, and possible ion-mediated bridging under alkaline conditions [9,14].

Because alkalinity can alter both chromophore state and tissue charge, pH is part of the active reagent specification rather than a processing detail. Excess hydroxide may weaken residual hematoxylin, increase background, or accelerate curcumin degradation in aqueous solution [9]. A publication-quality kumkum protocol should specify the *Curcuma* species, plant part, curcuminoid marker concentration, calcium hydroxide concentration, final pH, preparation time, filtration, particle burden, and reagent age. Findings obtained with a defined herbal formulation cannot be generalized to retail kumkum of unknown composition [8,14-16].

Henna

Henna leaves contain lawsone, or 2-hydroxy-1,4-naphthoquinone, as the principal orange-red chromophore, together with tannins, flavonoids, sugars, and other phenolics. Lawsone is a relatively small planar aromatic molecule containing quinone carbonyl groups and a phenolic hydroxyl group. These features permit hydrogen bonding, dipole interactions, hydrophobic association with proteinaceous domains, and redox-dependent reactions. Henna preparations are nevertheless mixtures, and the quantity of extractable lawsone depends on cultivar, leaf age, grinding, extraction solvent, pH, temperature, and storage [13,37].

The persistent coloration of keratinized substrates is commonly attributed to lawsone interaction with keratin and other proteins. In fixed sections, plausible retention mechanisms include diffusion into protein-rich matrices, hydrogen bonding to peptide and side-chain groups, hydrophobic and pi-associated interactions with aromatic residues, and possible nucleophilic addition of protein amino or thiol groups to the electrophilic quinone system. The last mechanism is chemically plausible but should not be presented as proven for formalin-fixed histological sections without direct spectroscopic or blocking evidence. Experimental work also shows that lawsone can bind a protein receptor through hydrogen-bonding and hydrophobic contacts, supporting its general capacity for noncovalent protein association [37].

Raju et al. reported that *Lawsonia inermis* extract could act as an eosin substitute in normal oral mucosa and oral squamous cell carcinoma, although the resulting color balance was not identical to H&E [13]. Deepthi et al. found henna to be the best-performing botanical counterstain among the candidates examined, with a mean composite score approaching the eosin control [8]. Lawsone can also penetrate human skin in vitro [38], and biologic effects have been demonstrated in keratinocyte systems [37]; these observations reinforce the need for exposure assessment rather than an assumption of safety based on traditional use. Studies should quantify lawsone, screen for para-phenylenediamine or other adulterants, and measure both working-solution stability and mounted-slide retention.

Ginger

Ginger rhizome contains a complex mixture of phenolic ketones and related compounds, principally gingerols, shogaols, paradols, and zingerone, as well as diarylheptanoids, volatile terpenes, carbohydrates, and proteins. Fresh ginger is generally richer in gingerols, whereas drying or heating promotes dehydration to shogaols; extraction polarity changes the relative recovery of pungent phenolics and volatile constituents. A recent gas chromatography-mass spectrometry (GC-MS) characterization of an ethyl acetate fraction identified zingerone and 6-gingerol as major constituents, illustrating the strong dependence of extract composition on source and fractionation [39].

The exact ginger constituent responsible for histological coloration has not been isolated. Phenolic hydroxyl and carbonyl groups can participate in hydrogen bonding and dipole interactions with tissue proteins, while the aromatic and hydrocarbon portions of gingerols and shogaols favor hydrophobic association. Deprotonated phenolic groups could contribute electrostatic attraction to cationic protein sites under appropriate pH conditions. Oxidation and polymerization of phenolics may also generate darker products

that adsorb nonspecifically. These are proposed mechanisms; they should be tested using purified 6-gingerol, 6-shogaol, and zingerone, marker-depleted fractions, and competitive or chemical-blocking experiments.

Ajileye et al. demonstrated staining of muscle fibers and cytoplasm with an ethanolic *Zingiber officinale* extract [40]. In oral tissues, Sudhakaran et al. found ginger closer to eosin than turmeric for intensity, specificity, background, and shelf-life measures [3], whereas Deepthi et al. reported a substantially lower composite score for ginger than for eosin [8]. The divergent results probably reflect differences in plant origin, drying, solvent, heating, pH, concentration, and scoring. A reproducible ginger reagent therefore requires a chromatographic or spectroscopic fingerprint and at least one quantitative marker rather than identification by plant name alone [3,8,39,40].

Onion skin and sorghum

Red or brown onion skin is rich in quercetin and quercetin glycosides, with anthocyanins contributing to red cultivars. Sorghum seed coats contain anthocyanins, condensed tannins, and other phenolics whose abundance varies widely by genotype. Crude extracts of *Allium cepa* skin and *Sorghum bicolor* seed have been reported to provide cytoplasmic staining in animal tissues [41]. Onion-skin extraction studies demonstrate that solvent composition, mass-to-liquid ratio, time, and temperature materially alter quercetin recovery [42], variables that are directly relevant to stain standardization.

Flavonol and tannin hydroxyl groups can form multiple hydrogen bonds with tissue proteins; aromatic surfaces can participate in hydrophobic interactions, and oxidized polyphenols may undergo stronger association with nucleophilic protein groups. Tannins are multidentate molecules capable of precipitating or cross-linking proteins, which may increase retention but can also produce coarse background and obscure fine morphology. Anthocyanin fractions add pH-dependent electrostatic and metal-coordination behavior. Because crude onion and sorghum extracts contain several interacting pigment families, fractionation and protein-binding assays are needed before assigning selectivity to a single compound [41,42].

Saffron

Saffron stigmas contain the water-soluble carotenoid glycosides crocins, their aglycone crocetin, the bitter glycoside picrocrocin, and the volatile compound safranal. The extended polyene system of crocin and crocetin produces yellow-to-orange color, while terminal carboxylate groups in crocetin and sugar-rich crocins influence polarity and tissue diffusion [43]. In an optimized hematoxylin-eosin-saffron method, alcoholic saffron produced bright yellow-to-orange collagen staining and improved contrast between connective tissue and muscle across several tissues [44].

Saffron retention in collagen has been attributed to dye size, diffusion, tissue porosity, and acidic dye behavior. Large crocin-rich molecules may accumulate preferentially within the hydrated, porous collagen network through physical entrapment and numerous weak hydrogen-bonding interactions. Crocetin carboxyl groups can also support electrostatic attraction to protonated amino groups, although the contribution of each saffron constituent has not been directly resolved in fixed tissue. Saffron is therefore more appropriately considered a connective-tissue component of a multichromatic stain than a complete replacement for H&E [43,44].

Annatto, paprika, and additional candidates

Annatto contains the apocarotenoids bixin and norbixin, while paprika contains capsanthin, capsorubin, β-carotene, and related carotenoids. These pigments are more hydrophobic than anthocyanins and generally require alcoholic, acetone-containing, or other organic extraction systems; norbixin is comparatively more polar because of its carboxyl groups. Proposed tissue retention depends mainly on nonpolar association, dispersion forces, and physical partitioning into hydrophobic protein domains, with possible ionic interactions for carboxylated pigments. These candidates remain exploratory, and many reports do not chemically identify the working stain [2].

Future screening should name the species, cultivar, plant part, and principal marker rather than using broad labels such as flower extract or natural dye. Each candidate should be tested as a single-component extract before mixtures are evaluated, with solvent-only, modifier-only, and purified-marker controls. UV-visible spectra, chromatographic fingerprints, pH, concentration, and post-dehydration retention should be correlated with blinded tissue scores. This approach can distinguish true tissue affinity from transient coloration, precipitate, oxidation products, or physical trapping. Table *2* summarizes representative published staining studies, including their materials, protocols, tissue models, findings, and interpretive limitations. The evidence should be compared in light of these methodological differences rather than treated as a uniform ranking of botanical stains.

Material and protocol	Tissue and design	Key findings	Interpretive limitation	References
Fractionated Curcuma longa; multiple solvents and mordants	Experimental histological staining	Selected fractions stained collagen and red blood cells; 70% ethanol supported useful staining.	No paired diagnostic-equivalence endpoint; chemical identity of active fractions was incomplete.	[4]
Curcuma longa extracts at varied concentrations	Multiple tissue samples	Demonstrated concentration-dependent histological staining potential.	Limited clinical validation and no multicenter reproducibility.	[5]
Alcoholic ginger and turmeric after hematoxylin	25 oral cases; coded evaluation and repeat review	Ginger generally showed better cytoplasmic specificity and shelf life; turmeric showed golden-yellow staining and more fading.	Small case set; categorical outcomes; not powered for diagnostic noninferiority.	[3]
Aqueous and ethanolic beetroot; spectrophotometry	Multiple albino-rat tissues	Stained muscle, mucin, red cells, keratin, and nerve fibers; extraction altered pigment characteristics.	Animal tissues; no diagnostic concordance or long-term slide validation.	[6]
Pomegranate anthocyanin with acid and storage modifications	Exploratory FFPE oral-tissue staining	Magenta staining of epithelial, basement-membrane, inflammatory, and collagen components.	Optimization study with limited quantitative and diagnostic endpoints.	[7]
Curcuma aromatica-slaked lime kumkum	Observational oral-tissue comparison	Reported differential staining of oral tissues and potential as a natural counterstain.	Formulation-specific; independent external replication remains limited.	[14]
Beetroot and turmeric compared with H&E	Laboratory comparison	Individual extracts showed staining potential, but the combination did not reproduce H&E quality.	Limited standardization and descriptive analysis.	[25]
Henna, ginger, turmeric, kumkum, hibiscus, and beetroot vs. eosin	140 sections from normal mucosa, OSCC, ameloblastoma, and odontogenic cysts; two observers	Eosin scored highest; henna was closest, kumkum intermediate, and beetroot/hibiscus lowest; ICC was 0.96.	Single center; composite scoring; long-term stability and true diagnostic noninferiority not established.	[8]
Curcumin in aqueous solutions and emulsions under varied pH and temperature	Physicochemical stability study	Curcumin stability depended on pH, temperature, and molecular environment; hydrophobic environments improved protection.	Not a histological study, but directly informs formulation and storage controls.	[9]
Roselle anthocyanins complexed with aluminum	Spectrophotometric formulation and histological evaluation	Produced a violet-blue nuclear-selective stain without prior oxidation and explained pH-dependent complexation.	Specific to a controlled roselle-metal complex rather than crude anthocyanin extracts generally.	[10]
Encapsulated beetroot betalains across pH 2-11	Absorption spectroscopy, HPLC, and LC-MS stability study	Individual betalains differed in pH stability; strongly alkaline storage caused substantial degradation of major pigments.	Food-colorant system, not FFPE tissue; histological retention still requires testing.	[11]

TABLE 2: Representative published evidence on botanical histological stains

Study-specific outcomes cannot be directly ranked.

FFPE, formalin-fixed paraffin-embedded; H&E, hematoxylin and eosin; HPLC, high-performance liquid chromatography; ICC, intraclass correlation coefficient; LC-MS, liquid chromatography-mass spectrometry; OSCC, oral squamous cell carcinoma.

Illustrative observations from a teaching laboratory exercise

The observations described here arose from a student laboratory exercise rather than a planned clinical or diagnostic-validation study. According to the authors, an author supplied buccal cells from their own mouth by cheek swab during the exercise. The specimens were collected cells, not archived oral-tissue biopsies. The classroom comparison involved routine H&E and three botanical preparations: beetroot-turmeric,

pomegranate, and blueberry-kumkum. The available record contains summary staining scores, but does not establish the number of independent donors, preparations, slides, or fields evaluated. These observations are presented only as an illustration of laboratory teaching and the limitations of exploratory stain comparisons.

Table 3 reproduces the recorded classroom scores for eight appearance or stability domains: nuclear staining, cytoplasmic staining, contrast, stability, background, clarity, intensity, and overall appearance. The four-point scale was 0, negative; 1, poor; 2, good; and 3, excellent. Each stain-domain combination has one summary score rather than replicate-level measurements. Means, variability, confidence intervals, statistical significance, and interobserver agreement cannot be estimated from these entries. An environmental rating in the source material is omitted because it was not supported by measured safety or sustainability outcomes.

Stain	Nuclear	Cytoplasm	Contrast	Stability	Background	Clarity	Intensity	Overall
Beetroot-turmeric	1	1	0	0	1	1	1	1
Blueberry-kumkum	1	0	0	0	0	0	0	0
Pomegranate	1	1	0	1	1	1	1	1
H&E	3	3	3	3	3	3	3	3

TABLE 3: Descriptive staining scores recorded during the teaching laboratory exercise

Source: authors' records of a student laboratory exercise using cheek-swab buccal cells. Scores: 0, negative; 1, poor; 2, good; 3, excellent. Scores are reproduced as recorded and are not replicate-level means or validated performance endpoints. Donor and replicate counts, blinding, observer agreement, and variability are not established. The direction of the background score and definition of stability require confirmation. "Clarity" and "Overall" retain the original record labels. The unmeasured environmental rating has been omitted.

H&E, hematoxylin and eosin

The recorded beetroot-turmeric scores were poor for nuclear staining, cytoplasmic staining, background, clarity, intensity, and overall appearance, and negative for contrast and stability. These entries describe the classroom record; they do not establish nuclear specificity, tissue binding, or diagnostic acceptability. Because the preparation combined two botanical ingredients, their individual contributions cannot be distinguished.

Pomegranate received recorded scores of poor for nuclear staining, cytoplasmic staining, stability, background, clarity, intensity, and overall appearance, and negative for contrast. Published anthocyanin-staining studies provide a rationale for further formulation work [7], but the classroom scores do not demonstrate tissue-boundary definition, chromatin preservation in biopsies, or diagnostic utility.

Blueberry-kumkum received a recorded poor nuclear score and negative scores for the other appearance and stability domains. This classroom result does not establish that either ingredient is intrinsically ineffective. Potential explanations such as pigment degradation, inappropriate pH, antagonistic mixing, or insufficient concentration would need testing under a documented protocol. Future exploratory work should assess each component separately before testing mixtures.

H&E received the highest recorded scores in all eight retained domains. Without defined replication, blinded assessment, and a validated scoring rubric, these entries support only a descriptive account of the exercise. They do not quantify comparative efficacy, reproducibility, diagnostic performance, or sustainability.

Mechanistic explanations cannot be derived from these classroom scores. Pigment concentration, ionization, degradation, formulation chemistry, and nonspecific adsorption are possible contributors to differences in cellular coloration, but their roles would require appropriately controlled experiments [7,10]. Cell-smear observations cannot demonstrate staining of collagen, stroma, basement membranes, or other architectural features of intact tissue. The published tissue-staining literature and the future validation framework therefore remain the basis for the histopathological discussion.

The record also has limitations in scoring definitions. "Background" could mean either greater unwanted coloration or better background quality. "Stability" could refer to reagent shelf life, short-term stain retention, or mounted-slide color. "Clarity" and "Overall" may overlap. A future study should define each endpoint, the direction of benefit, preparation type, magnification, number of fields and replicates, scoring anchors, and observer blinding before evaluation. The teaching exercise should not be used as a substitute for that study.

Methodological sources of variability

Botanical Identity and Preanalytical Material

Species, cultivar, plant part, maturity, geographic origin, harvest season, drying, grinding, transport, and storage influence pigment concentration and co-extracted compounds. Commercial powders may contain excipients, adulterants, preservatives, or synthetic dyes. Botanical authentication, voucher documentation where feasible, supplier and lot traceability, moisture content, and acceptance specifications are therefore necessary. Independent batches should be tested to separate true stain performance from a favorable single lot [2,8].

Extraction and Formulation

Water, ethanol, methanol, mixed solvents, heating, maceration time, particle size, mass-to-volume ratio, filtration, centrifugation, and concentration determine pigment yield. Extracts should be characterized by pH and absorbance spectrum at minimum; chromatography is preferable when a defined marker can be quantified. Reports should state the final pigment or marker concentration, solvent composition, preparation yield, filtration method, storage container, light protection, temperature, and allowable age. Without these details, another laboratory cannot reproduce the reagent [3-7].

pH, Mordants, Differentiation, and Interactions

Ionization governs electrostatic interaction between dyes and tissue. Anthocyanin hue changes sharply with pH, curcuminoid solubility is solvent-dependent, and betalains degrade outside favorable processing conditions. Alum, iron salts, acetic acid, lemon extract, vinegar, lime, and other modifiers can alter binding or color, but may also affect hematoxylin or tissue morphology. Factorial optimization should vary one or a limited set of prespecified parameters and include interaction terms, rather than altering extraction, acidity, staining time, and mordant simultaneously [3,6,7].

Fixation, Processing, Sectioning, and Staining Workflow

Cold ischemia, fixation type and duration, paraffin processing, block age, section thickness, drying, deparaffinization, hydration, rinse-water chemistry, dehydration, clearing, and mounting can all affect uptake or pigment retention. Serial sections from the same block reduce biological variability but are not microscopically identical; randomized assignment of nearby sections and replicate staining runs are preferable. Candidate protocols should be tested in keratinized and nonkeratinized mucosa, salivary tissue, muscle, inflammation, dysplasia, benign lesions, and carcinoma because tissue composition changes staining behavior [3,8].

Reagent Age, Slide Stability, and Laboratory Environment

A stain may perform well when freshly prepared yet fail during routine storage. Reagent stability should be evaluated prospectively at defined temperatures and light conditions using predefined acceptance ranges for pH, absorbance, contamination, precipitate, and staining score. Mounted slides should be reassessed after days, weeks, and months under controlled storage. Temperature, humidity, water quality, and instrument carryover should be logged because they can confound between-run comparisons [3,8].

Observers, Imaging, and Statistical Design

Knowledge of the stain can influence qualitative scoring, particularly when botanical colors are distinctive. Slides should receive concealed random codes, be presented in randomized order, and be assessed independently by multiple calibrated oral pathologists. A washout period is needed if the same observer evaluates paired sections. Prespecified anchors should define each score, and both weighted kappa or intraclass correlation and confidence intervals should be reported. Digital imaging requires fixed illumination, white balance, exposure, magnification, scanner profile, and color calibration. Optical density, color difference, texture, and color-deconvolution measures may complement, but not replace, diagnostic assessment.

Mechanistic and Analytical Characterization

A development study should link tissue performance to chemical measurements. UV-visible spectra can monitor wavelength maxima, spectral shape, and degradation; HPLC with diode-array detection can quantify curcuminoids, betalains, or anthocyanins; LC-MS can confirm marker identity and degradation products [9-11,34]. Working solutions should be tested for pH, conductivity, turbidity, precipitate, microbial burden, and marker concentration. On stained sections, region-specific optical density and CIELAB color coordinates can quantify hue and saturation, while color difference metrics can compare batches. These measurements should be correlated with blinded morphologic scores rather than used as surrogates for diagnostic adequacy.

Controls and Experimental Isolation

Appropriate controls are essential for mixtures and mordanted systems. Recommended controls include unstained tissue, hematoxylin-only tissue, each botanical component alone, solvent-only control, modifier-only control, and the complete formulation. When a metal mordant is used, a mordant-only section can reveal endogenous or nonspecific effects. A factorial design can estimate main effects and interactions among pigment concentration, pH, mordant, time, and temperature. This approach is more informative than sequential trial-and-error optimization and reduces the risk of attributing a result to the wrong ingredient. Figure *3* summarizes the proposed physicochemical interactions between plant pigments and fixed tissue, providing a conceptual basis for selecting these mechanistic controls.

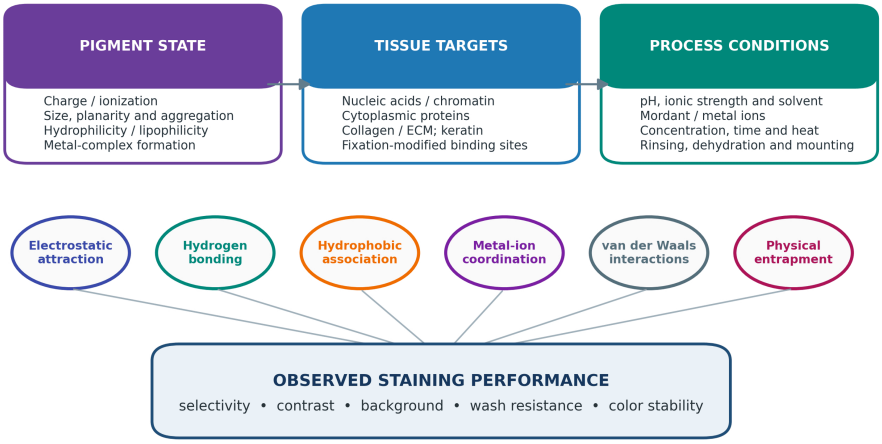


FIGURE 3: Proposed pigment-tissue interactions

This conceptual schematic was created using ChatGPT (OpenAI, San Francisco, CA, USA), a generative artificial intelligence (AI) tool to illustrate the relationships discussed in the review. It does not represent experimental measurements or microscopy data. Proposed interactions between botanical pigments and formalin-fixed tissue. Pigment ionization, molecular size, hydrophobicity, and metal-complex formation may influence association with nucleic acids, proteins, collagen, ECM, and keratin. Electrostatic attraction, hydrogen bonding, hydrophobic association, metal-ion coordination, van der Waals interactions, and physical entrapment are candidate mechanisms. Their relative contributions require experimental testing for each formulation. These proposed mechanisms are informed by histological staining and pigment-binding literature [1,10,20,22,23,26].

ECM, extracellular matrix

Chemical structure atlas of stain chromophores

The following structure atlas identifies the chemically defined chromophores represented by the stains discussed in this review. Plant extracts and manufactured preparations are multicomponent systems and therefore do not possess a single chemical structure. The figures show principal or representative marker compounds rather than asserting that one molecule alone produces the observed tissue color. Structures, molecular formulas, and compound identifiers were standardized against PubChem records [19]. Table *4* maps each stain source to its representative marker compounds. Figure *4* depicts the H&E reference chromophores, Figure *5* depicts representative betalains and anthocyanins, and Figure *6* shows marker compounds for henna. Figure *7* shows representative saffron, annatto, and paprika pigments. These panels complement the curcuminoid structure in Figure *2*.

Stain or source discussed in the manuscript	Representative structure or marker shown	References
Routine H&E reference	Hematoxylin, its oxidized chromophore hematein, and eosin Y disodium	[1,19]
Turmeric and curcuminoid stains	Curcumin, demethoxycurcumin, and bisdemethoxycurcumin (Figure 2)	[12,17-19]
Kumkum	No single structure; traditionally a curcuminoid-containing alkaline preparation. Curcuminoids are represented in Figure 2; calcium hydroxide is an inorganic formulation component.	[9,12,14-16]
Beetroot	Betanin as a representative red-violet betacyanin and vulgaxanthin I as a representative yellow betaxanthin	[6,11,19]
Pomegranate	Cyanidin-, delphinidin-, and pelargonidin-3-glucosides as representative aril anthocyanins	[7,19,27,33,34]
Blueberry	Mixtures of malvidin-, delphinidin-, petunidin-, cyanidin-, and peonidin-based glycosides. Figure 5 shows selected anthocyanin markers rather than a complete blueberry pigment profile.	[19,26,28]
Hibiscus and roselle	Cyanidin- and delphinidin glycosides; the exact sugar substitution depends on species and preparation	[10,19,26,35]
Rose petals and black rice	Cyanidin-3-glucoside; peonidin-3-glucoside is additionally shown for black rice	[19,26,29,32]
Mulberry	Cyanidin-3-rutinoside and cyanidin-3-glucoside	[19,26,30]
Red cabbage	Acylated cyanidin glycosides; no single universal structure because glycosylation and acylation patterns vary	[10,19,26,31]
Henna	Lawsone	[13,19,37,38]
Ginger	6-Gingerol, 6-shogaol, and zingerone	[3,19,39,40]
Onion skin	Quercetin as a principal flavonol marker	[19,41,42]
Sorghum	Luteolinidin and apigeninidin as representative 3-deoxyanthocyanidins; tannins are heterogeneous polymers	[19,41]
Saffron	Crocin and its aglycone crocetin	[19,43,44]
Annatto	Bixin and norbixin	[2,19]
Paprika	Capsanthin and capsorubin	[2,19]

TABLE 4: Botanical sources and representative chemical markers

Marker compounds represent selected constituents, not complete extract composition. Figure 2 and Figures 4-7 depict selected structures. Salt forms, ionization, glycosylation, and source composition may vary.

CID, compound identifier; H&E, hematoxylin and eosin

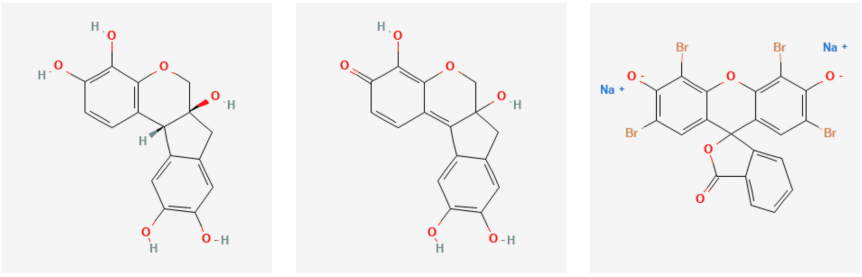


FIGURE 4: Reference H&E chromophores

Chemical structure images were downloaded from the corresponding PubChem compound records and assembled into a panel in Microsoft PowerPoint (Microsoft Corp., Redmond, WA, USA), then exported as a JPG for submission. The chemical structure depictions were obtained from PubChem and were not generated using AI. Chemical structures of hematoxylin, hematein, and eosin Y disodium used to contextualize the reference H&E system. Hematoxylin is oxidized to hematein, which forms a metal-mordanted nuclear-staining complex. Eosin Y is represented as the disodium salt. The free chromophores shown do not represent the complete coordination chemistry of the working H&E reagents. Compound identities are referenced to PubChem records; the PubChem database is described by Kim et al. [19].

CID, compound identifier; H&E, hematoxylin and eosin

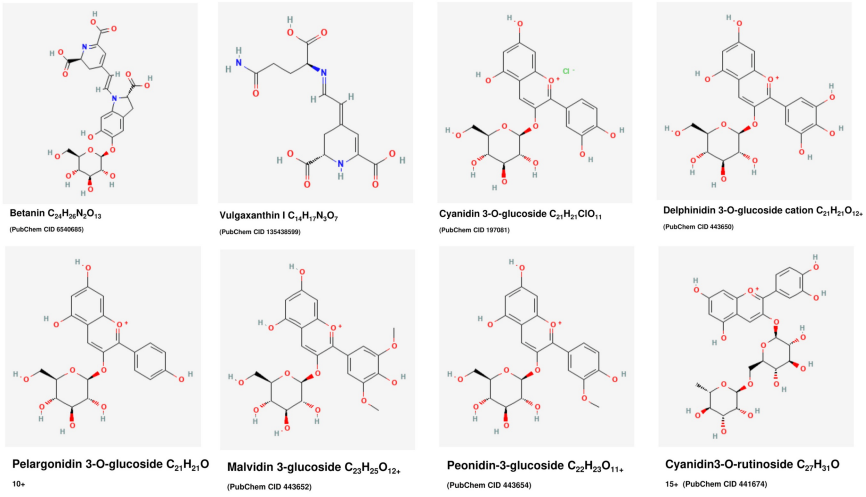


FIGURE 5: Betalain and anthocyanin marker structures

Chemical structure images were downloaded from the corresponding PubChem compound records and assembled into a panel in Microsoft PowerPoint (Microsoft Corp., Redmond, WA, USA), then exported as a JPG for submission. The chemical structure depictions were obtained from PubChem and were not generated using AI. Representative betalain and anthocyanin marker structures. Betanin represents red-violet betacyanins, and vulgaxanthin I represents yellow betaxanthins. The anthocyanin panel includes cyanidin-, delphinidin-, pelargonidin-, malvidin-, and peonidin-3-glucosides, together with cyanidin-3-rutinoside. Molecular formulas and PubChem compound identifiers correspond to the molecular or salt forms depicted; counterions are not uniform across the panel. These markers do not describe the complete pigment composition of any botanical extract. Structures alone do not demonstrate fixed-tissue binding. Compound identities are referenced to PubChem records; the PubChem database is described by Kim et al. [19].

CID, compound identifier

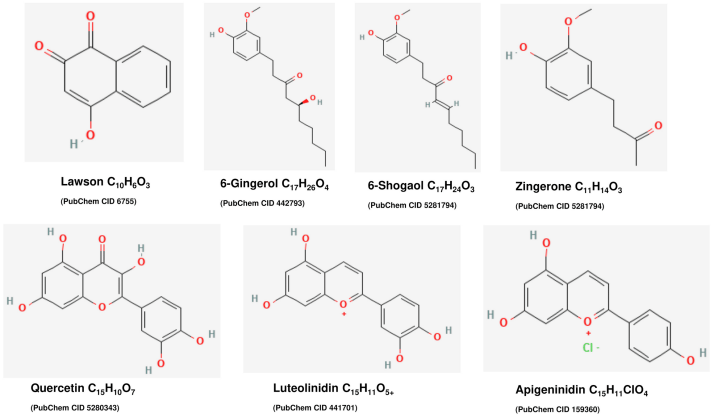


FIGURE 6: Henna, ginger, onion, and sorghum marker structures

Chemical structure images were downloaded from the corresponding PubChem compound records and assembled into a panel in Microsoft PowerPoint (Microsoft Corp., Redmond, WA, USA), then exported as a JPG for submission. The chemical structure depictions were obtained from PubChem and were not generated using AI. Representative marker structures for henna, ginger, onion skin, and sorghum. The panel shows lawsone, 6-gingerol, 6-shogaol, zingerone, quercetin, luteolinidin, and apigeninidin. Molecular formulas and PubChem identifiers refer to the forms depicted. Extracts also contain other phenolics and nonchromophoric constituents; the presence of a marker does not establish that it is the sole cause of tissue coloration. Compound identities are referenced to PubChem records; the PubChem database is described by Kim et al. [19].

CID, compound identifier

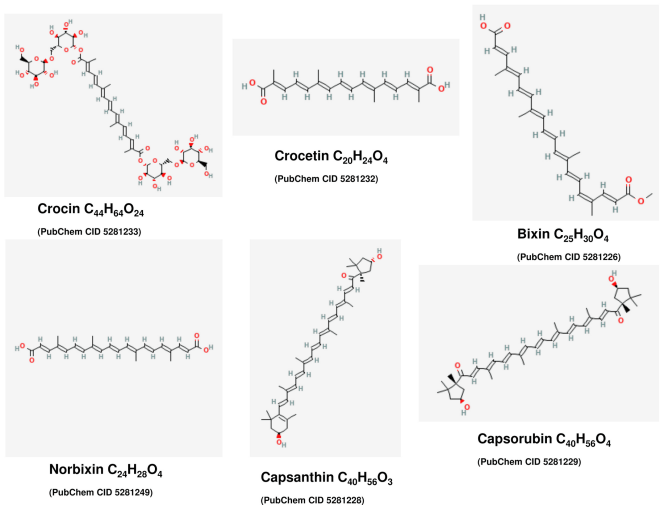


FIGURE 7: Saffron, annatto, and paprika marker structures

Chemical structure images were downloaded from the corresponding PubChem compound records and assembled into a panel in Microsoft PowerPoint (Microsoft Corp., Redmond, WA, USA), then exported as a JPG for submission. The chemical structure depictions were obtained from PubChem and were not generated using AI. Representative saffron, annatto, and paprika pigments: crocin and crocetin; bixin and norbixin; and capsanthin and capsorubin, respectively. Molecular formulas and PubChem identifiers refer to the depicted structures. Glycosylation, esterification, cis-trans isomerism, oxidation, and extraction conditions can change the pigment profile of the working stain. Compound identities are referenced to PubChem records; the PubChem database is described by Kim et al. [19].

CID, compound identifier

Safety, sustainability, and cost

Natural origin does not establish low hazard. Extracts may contain allergens, irritants, pesticides, heavy

metals, microbes, mycotoxins, or undeclared additives. The whole workflow may still use flammable alcohol, corrosive acids, xylene, and DPX. Occupational assessment should address preparation aerosols, skin exposure, labeling, storage, spills, microbial preservation, and disposal. Environmental claims require comparison of solvent use, water and energy demand, packaging, transportation, waste classification, reagent shelf life, repeat-slide frequency, and end-of-life disposal.

Economic evaluation should use functional outcomes such as cost per acceptable slide or cost per correctly interpretable case. Raw plant material may be inexpensive, yet labor-intensive extraction, quality testing, refrigeration, short shelf life, prolonged staining, technical failures, and repeat sections may eliminate the apparent advantage.

Safety characterization should follow the actual exposure route. Inhalation of dry powders, dermal contact with concentrated extracts, solvent vapor, and splashes to the eyes may pose different risks. Lawsone, for example, can penetrate human skin in vitro, illustrating why traditional use does not remove the need for exposure assessment [38]. Sustainability analysis should define a functional unit and system boundary; otherwise, comparisons can be distorted by excluding cultivation, drying, cold storage, solvent recovery, or repeat-slide burden. Table *5* organizes these translation requirements by development domain and identifies the evidence needed to establish a controlled laboratory reagent.

Development domain	Required evidence and recommended acceptance elements	Reference
Botanical identity and supply	Authenticated species and plant part; supplier qualification; lot and geographic traceability; controls for adulteration, moisture, pesticides, heavy metals, and microbial contamination; retained samples and change notification.	[2,8]
Chemical characterization	Marker-pigment identity and concentration; absorbance or chromatographic fingerprint; pH, solvent, and buffer specifications; impurity profile; extraction yield; predefined batch-release ranges.	[9-12,17,18,34]
Formulation and reagent stability	Locked formula and preparation instructions; container and light protection; opened and unopened shelf life; temperature excursions; preservative effectiveness where applicable; acceptance testing over time.	[3,9-11,17,21]
Analytical staining performance	Selectivity, intensity, contrast, background, uniformity, reproducibility, robustness, carryover, between-run and between-laboratory variation, and mounted-slide color retention using predefined criteria.	[1,3,8]
Diagnostic performance	Blinded paired comparison with H&E across representative normal and pathological tissues; diagnostic concordance, sensitivity for key morphologic features, confidence, failure rate, and justified noninferiority margin.	[1,3,8]
Observer and image controls	Pathologist training and calibration; concealed codes; randomized reading order; washout; inter- and intraobserver agreement; standardized imaging and objective color or texture measurements.	[1,8]
Workflow and human factors	Clear instructions, extraction and staining time, operator variability, equipment compatibility, throughput, troubleshooting, training needs, and documented quality-control slides.	[1-3,8]
Safety and sustainability	Chemical and microbiological hazards; worker exposure; SDS and labeling; solvent, water, energy, waste, packaging, and disposal measurements; substantiated environmental claims.	[2,37,38]
Quality system and implementation	Controlled specifications, batch records, deviations, CAPA, change control, proficiency testing, lot bridging, ongoing QC trending, and defined criteria for rejecting reagent or stained slides.	[1,2]
Clinical-use claim	Claim limited to the evidence generated: adjunct, teaching, contingency, or replacement. Replacement requires diagnostic noninferiority and external validation, not solely similar coloration.	[1,8]

TABLE 5: Evidence framework for botanical stain development

CAPA, corrective and preventive action; H&E, hematoxylin and eosin; QC, quality control; SDS, Safety Data Sheet

These domains are interdependent. Chemical specifications permit meaningful lot comparison; analytical staining studies establish robustness; diagnostic studies determine clinical utility; and the quality system maintains performance after implementation. Failure at an early stage cannot be compensated by a favorable mean score in a small observer study. In particular, a formulation should not enter a diagnostic noninferiority study until identity, concentration, pH, stability, and staining instructions have been locked.

Framework for future confirmatory validation

Study Objective and Design

A future prospective, paired, blinded diagnostic-performance study could compare a locked botanical protocol with routine H&E on adjacent sections from archived formalin-fixed, paraffin-embedded oral-tissue blocks. This is a proposed tissue study, distinct from the cheek-swab teaching exercise, and has not been conducted. Candidate selection should follow a separate optimization phase informed by the published literature; mixture components should be assessed individually. Institutional ethics review or an applicable determination, tissue-access authorization, and any required consent or waiver must be addressed before the proposed work begins. The protocol, scoring rubric, analysis plan, and exclusion rules should be finalized before evaluation.

Sample-Size Planning

An illustrative design could target 120 evaluable cases distributed across six lesion groups: normal or reactive mucosa, inflammatory lesions, benign epithelial or mesenchymal lesions, potentially malignant disorders or epithelial dysplasia, oral squamous cell carcinoma, and salivary or other diagnostically distinct oral lesions. A proposed allocation of 20 cases per group is a planning example, not a validated sample-size calculation. The final paired design requires a formal calculation using justified acceptability assumptions, a clinically defensible noninferiority margin, within-case discordance, desired power, and expected exclusions. These inputs must come from suitable tissue-study evidence or a separately authorized feasibility study, not from the classroom summary scores.

Blinded Evaluation

Two or preferably three board-certified oral pathologists should evaluate independently coded slides in randomized order. Botanical and H&E sections from the same case should not appear in the same reading session. A washout interval of at least two weeks should separate methods. Stain identity should be concealed as far as color permits, and analysts should remain blinded until the database is locked. Ten to fifteen percent of slides should be reread to estimate intraobserver reliability.

Outcomes

The primary outcome should be the proportion of slides rated diagnostically acceptable, defined in advance as sufficient to identify the principal lesion category and assess key nuclear, cytoplasmic, epithelial, stromal, and inflammatory features without restaining. Key secondary outcomes should include lesion-category concordance with the reference diagnosis; nuclear detail; cytoplasmic detail; contrast; background; keratin and collagen visualization; pathologist confidence; technical failure and repeat-stain rates; preparation and staining time; cost per acceptable slide; reagent stability; and mounted-slide color retention at baseline, 1 month, three months, and six months.

Statistical Analysis

The primary paired difference in acceptability should be reported with a two-sided 95% confidence interval; noninferiority is concluded only if the lower confidence bound is above -10%. Paired binary outcomes should be analyzed with McNemar methods, and ordinal scores with mixed-effects ordinal regression or an appropriate paired nonparametric analysis. Models should include stain, lesion category, observer, and relevant interactions, with case as a random effect. Diagnostic concordance should be summarized with percentage agreement and weighted kappa; continuous image measures should use paired mean differences or robust alternatives with confidence intervals. Missing or technically failed slides should be tabulated by reason, and the primary analysis should conservatively count non-evaluable botanical slides as failures. Multiplicity should be controlled for key secondary outcomes, while exploratory outcomes should be labeled accordingly.

Preanalytical and Quality Controls

Adjacent sections should be assigned to stain arms according to a predefined alternating sequence, and section thickness should be verified periodically. Each run should include a control block containing epithelium, connective tissue, muscle, and blood vessels where feasible. Botanical reagent lots and staining runs should be treated as design factors, with at least three independent reagent preparations and multiple days represented. Scanner calibration and image-acquisition settings should be fixed before analysis. Protocol deviations, restains, exclusions, and adverse handling events should be documented before unblinding.

Interpretation Plan

Noninferiority in overall acceptability should not override a clinically important loss of nuclear detail or

lesion-specific performance. Results should therefore be stratified by lesion group, keratinization, inflammation, and tissue compartment. A successful confirmatory study would justify external replication, not immediate replacement of H&E. Multicenter validation should include different processors, water supplies, operators, and scanners to test transportability.

Research priorities

The first priority is to move from named plants to defined reagents. Published discrepancies for turmeric, ginger, beetroot, hibiscus, and kumkum show that botanical identity alone does not predict staining performance [3,6,8]. Investigators should identify marker pigments, set concentration and pH specifications, document extraction yield, and demonstrate consistency across at least three independently prepared lots. Studies should publish complete protocols and raw or case-level outcome data so that external laboratories can reproduce the work.

Second, optimization and confirmation should be separated. Screening experiments may explore solvent, concentration, mordant, acidity, time, and temperature, but the selected formulation must then be locked before blinded testing. Pomegranate and other anthocyanin preparations particularly require pH and stability controls [7,33], while betalain studies require protection from heat, light, oxygen, and processing-related loss [6]. Mixtures such as beetroot-turmeric or blueberry-kumkum should be evaluated by component and factorial combination to determine whether effects are additive, antagonistic, or simply due to one ingredient.

Third, future studies should measure diagnostic utility. Existing reports have largely emphasized color intensity and morphologic appearance [3-8,14]. Representative lesion spectra, paired sections, blinded readers, prespecified diagnostic-acceptability criteria, confidence intervals, agreement statistics, and technical-failure reporting are needed. Multicenter validation is essential because local water, processing, laboratory temperature, scanners, and observer training may influence results.

Fourth, durability, safety, sustainability, and cost require direct measurement rather than assumption. Sudhakaran et al. showed that fading and solution longevity can distinguish otherwise plausible candidates [3]. Studies should therefore evaluate reagent shelf life and mounted-slide retention under controlled conditions. Toxicological screening, contaminant controls, solvent accounting, waste characterization, and cost per acceptable slide should accompany performance testing. Only after these steps should investigators consider claims for adjunctive, contingency, or replacement use.

A fifth priority is mechanistic development. The demonstration that aluminum complexation can transform roselle anthocyanins into a nuclear-selective stain shows that candidate performance can be engineered through molecular stabilization rather than accepted as an immutable property of a crude extract [10]. Similar work should examine curcuminoid chelation, betalain degradation pathways, copigmentation, buffer systems, and polymeric stabilizers [9-11]. Structure-performance relationships could yield defined plant-derived reagents with greater selectivity and shelf life than raw extracts.

A sixth priority is transparent reporting and data sharing. Minimum reporting should include botanical authentication, extraction yield, complete formula, marker concentration, pH, absorbance profile, tissue processing, section thickness, timing, temperature, wash conditions, mounting medium, observer training, scoring anchors, and raw case-level outcomes. Photomicrographs should use calibrated color and scale bars. Such reporting would permit systematic evidence synthesis and reduce repeated studies that cannot be compared or reproduced.

Interpretation and translational outlook

Plant-derived pigments offer a scientifically credible source of histological colorants, and published studies confirm that curcuminoids, betalains, anthocyanins, lawsone-containing henna, ginger extracts, and selected kumkum formulations can color diagnostically relevant tissue structures [3-8,14-16]. The evidence is nevertheless heterogeneous. The same botanical source may perform well in one protocol and poorly in another because extraction, concentration, pH, mordanting, tissue processing, evaluation criteria, and storage are not standardized. Most studies demonstrate staining feasibility rather than diagnostic equivalence. The separately described teaching exercise used cheek-swab buccal cells and illustrates why visual staining scores need carefully defined endpoints. It does not extend the evidence for diagnostic histopathology or validate the use of botanical stains in tissue sections.

The most defensible near-term role for botanical stains is as a platform for reagent development, education, adjunctive visualization, or carefully controlled contingency use. Transition to routine oral histopathology requires authenticated raw materials, chemically characterized and stable formulations, robust manufacturing controls, blinded paired evaluation, objective imaging, multicenter reproducibility, and clinically meaningful diagnostic endpoints. Until those requirements are met, H&E should remain the reference method and any botanical preparation should be presented as investigational rather than interchangeable. Scientifically, the field should shift from asking whether a plant can color tissue to determining which molecular species bind which tissue targets, under what physicochemical conditions,

with what durability, and with what effect on diagnosis. Curcumin instability, betalain degradation, anthocyanin structural equilibria, metal complexation, and lawsone exposure illustrate why chemical characterization and biological performance cannot be separated [9-11,34,38]. A successful botanical stain will therefore be a controlled reagent derived from a plant source, not merely a crude extract with an appealing color.

Conclusions

Plant-derived pigments are promising candidates for histological reagent development, but the available evidence does not establish that they can replace routine H&E in oral diagnostic practice. Extract composition, formulation, tissue processing, and storage influence their staining behavior. The illustrative classroom observations concern cheek-swab buccal cells and cannot establish tissue-staining performance, diagnostic equivalence, or environmental superiority. Progress requires chemically defined formulations, reproducible protocols, blinded assessment of diagnostic acceptability, and direct evaluation of stability, safety, sustainability, and cost. Botanical stains should undergo controlled validation before routine clinical use.

Additional Information

Author Contributions

All authors have reviewed the final version to be published and agreed to be accountable for all aspects of the work.

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