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Histological Evaluatıon of Fixation with Pine Honey and Propolis as an Alternative to Formalin in Oral Tissue

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ABSTRACT

Background: Formaldehyde-based fixatives, particularly 10% neutral buffered formalin (NBF), remain the reference standard in histopathology. However, the fact that formaldehyde is a Group 1 carcinogen has led to research into safer and more environmentally sustainable alternatives. This study aimed to evaluate the histomorphological preservation efficacy of propolis and pine honey as natural fixatives in oral tissues.

Methods: Sheep tongue and buccal mucosa specimens were fixed in 10% NBF, 10% propolis, 10% pine honey and a combination of 5% propolis + 5% pine honey for 24 hours. Tissue morphology was assessed using a semi-quantitative scoring system.

Result: The NBF group demonstrated optimal preservation across all evaluated parameters, with well-defined epithelial architecture, homogeneous nuclear chromatin and minimal stromal artifacts. Propolis provided moderate but diagnostically acceptable preservation, characterised by mild epithelial shrinkage and slightly reduced staining intensity. Pine honey showed comparable moderate preservation, with evidence of cytoplasmic shrinkage. The combination group demonstrated improved structural integrity compared to honey alone, demonstrating enhanced cellular boundary definition and basement membrane continuity. 10% NBF maintains its superiority in preserving histomorphological structure, while propolis, especially in combination with pine honey, provides good tissue preservation. These findings support the benefits of honey as a safer alternative for histological applications in research.

Key words: Fixation, Histomorphology, Pine honey, Propolis.

INTRODUCTION

In the realm of histopathology, fixation is essentially the “art of arresting time.” Fixation is a fundamental histological process that stabilises tissue specimens in a state as close as possible to their living form by preventing autolysis and bacterial putrefaction. This chemical intervention preserves structural integrity and provides the necessary mechanical rigidity required to obtain thin sections for precise microscopic examination. The primary objective of successful fixation is to maintain diagnostically critical morphological features, including nuclear detail, cytoplasmic structure, stromal organisation and basement membrane continuity (Ajileye and Esan, 2022; Fox *et al*., 1985). A histologically ideal fixative must not only preserve morphology but also facilitate the necessary hardening of the tissue to withstand the mechanical rigors of microtomy and the subsequent dehydration cycles of tissue processing (Fox *et al*., 1985; Bancroft and Gamble, 2008).

Since the 1890s, when Ferdinand Blum serendipitously discovered its preservative properties, 10% Neutral Buffered Formalin (NBF) has remained the undisputed “gold standard.” Its mechanism, the formation of methylene bridges between amino acid residues, provides the structural rigidity that histologists have relied upon for over a century (Bancroft and Gamble, 2008; Kiernan, 2015; Protano *et al*., 2021). However, the modern laboratory faces a significant ethical and safety crisis. Formaldehyde is now strictly classified as a Group 1 human carcinogen by the IARC, linked to nasopharyngeal malignancies and leukemia (Mohammed *et al*., 2019). This has fueled an urgent quest for “Green Histopathology”, the search for non-carcinogenic, bio-friendly agents that can achieve the same cross-linking precision as aldehydes without the associated toxic footprint.

Among the natural contenders, honey and propolis represent a fascinating biochemical alternative. Honey, an organic hyperosmolar matrix defined by the Codex Alimentarius, functions through a combination of high acidity, hygroscopicity and hydrogen peroxide, which collectively “mummify” the tissue at a cellular level (Protano *et al*., 2021; Kumar *et al*., 2019; Mohammed *et al*., 2019). Propolis, or “bee glue,” provides a complex resinous matrix of flavonoids and phenolic compounds known for their anti-autolytic properties (Alshehri, 2024; Genç *et al*., 2020; Thrasyvoulou *et al*., 2018; Bugshan *et al*., 2022; Oršolić and Jembrek, 2022). While their utility in dentistry is recognised, their efficacy in stabilising the dense, multidirectional skeletal

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muscle fibers and keratinised epithelium of the tongue a particularly challenging tissue due to its high protein density remains a subject of rigorous investigation (Bankova *et al*., 2014). The aim of this study was to evaluate the fixative efficacy of 10% propolis, 10% pine honey and a synergistic 5%+5% combination as non-toxic alternatives to the gold standard 10% neutral buffered formalin. By using sheep tongue mucosa as a complex tissue model, we seek to determine if these natural agents can sufficiently preserve diagnostic morphological features, specifically nuclear detail, cytoplasmic integrity and stromal architecture, while providing the mechanical rigidity necessary for routine histological processing.

MATERIALS AND METHODS

The *in vitro* study was conducted in the Histology Department Laboratory of the Faculty of Veterinary Medicine, Harran University, Şanlıurfa, Turkey. This study was conducted between October 2025/March 2026.

Study design

Two bee products, pine honey and propolis, were used as fixatives. The most important characteristic of pine honey is that it can be stored for years without deterioration or freezing. Its color is darker than most floral honeys. Propolis, on the other hand, is a bee product with a distinctive odor and a color that can vary from green to red and brown. In this study, we used 10% pine honey, 10% propolis, 5% pine honey + 5% propolis and 10% formalin groups as fixative concentrations. In this study, we selected experimental conditions such as temperature of 20-24°C, fixation time of 24 hours, tissue thickness of 3 mm and fixation volume of 1:10. This is because a fixation volume of 1:10 has been reported by many researchers and has been found to be the most recommended ratio (Zulhendri *et al*., 2021; Sabarinath *et al*., 2014).

Sample processing

Samples for the in vitro study were sheep oral tissues (tongue and buccal mucosa) remaining from commercially sold meat for human consumption in butcher shops. The tissues were collected immediately after the animal was slaughtered for meat production. Tissue samples brought to the laboratory were washed with saline. Fresh samples measuring 1 cm × 1 cm × 1 cm were immediately fixed by immersion in containers containing 10% pine honey, 10% propolis, 5% pine honey + 5% propolis and 10% formalin solution (Uppal *et al*., 2019), (37% v/v, Sigma-Aldrich, Germany), respectively.

Nine samples were fixed in each group: four uniform thickness samples (3 mm) were taken from the apex, corpus and radix lingua of the dorsal surface of the sheep tongue, four from the lateral tongue tissue and four from the buccal mucosa. They were trimmed with a microtome blade to obtain a uniform thickness for all groups. Tissues were immediately placed in fixatives at room temperature (18-23°C) for 24 hours. After fixation, the same procedure was applied to all tissues. Following fixation, all samples underwent identical processing procedures using an automated tissue processor (Leica TP1020, Leica Microsystems, Germany). The processing protocol included graded alcohol dehydration, xylene clearing and paraffin infiltration (Gur *et al*., 2017). Paraffin-embedded tissue blocks were sectioned at 6 µm thickness using a rotary microtome (Leica RM2135, Leica Microsystems, Germany). Sections were mounted on glass slides and stained with Mallory's triple staining technique to evaluate general histomorphological architecture.

Image analysis

A total of 200 slides were prepared for the formalin-fixed (F) and honey-fixed (H) groups, respectively. Slides were examined using a light microscope (Olympus BX51, Olympus, Japan) equipped with a digital photomicrographic system (DP71, Olympus, Japan).

Histomorphological assessment was performed using a semi-quantitative scoring system evaluating: epithelial integrity, nuclear detail, stromal organization, muscle fiber continuity, overall staining quality. Each parameter was scored on a 5-point scale:

0 = Very poor.
1 = Poor.
2 = Fair.
3 = Good.
4 = Excellent.

The final score for each specimen was calculated as the mean of all parameters.

RESULTS AND DISCUSSION

The histomorphological effects of various natural and conventional fixatives on the tongue (apex, corpus, radix, lateral) and buccal mucosa were evaluated. Our analysis focused on epithelial integrity, preservation of nuclear detail, stromal organization, muscle fiber continuity and overall staining quality using a semi-quantitative scoring system (Table 1).

10% neutral buffered formalin (Control group) histological observations

The control group fixed with 10% formalin demonstrated superior results in terms of tissue processing and sectioning. Specimens from all anatomical regions (apex, corpus, radix, lateral tongue and buccal mucosa) yielded high-quality sections with minimal tissue loss during staining. Histological examination revealed that the stratified squamous epithelium remained perfectly intact, with clearly defined lingual papillae (filiform and fungiform) and a continuous basement membrane. The nuclear chromatin distribution was homogeneous, allowing for sharp cellular detail. In the underlying connective tissue, we observed minimal artifacts, while the skeletal muscle fibers maintained a regular, parallel organization.

Significant pyknosis and karyorrhexis were noted in the nuclear detail. Epithelial integrity was assessed as complete, cellular boundaries as distinct and stromal organization as flawless. These findings confirm that formalin provides robust morphological stability through its established protein cross-linking mechanism (Fig 1).

10% pine honey group histological observations

When 10% pine honey was used as a fixative, tissue integrity was better preserved and blocks yielded better sections compared to formalin, propolis and chestnut honey. In the pine honey group, epithelial integrity was preserved, but superficial irregularities and stromal relaxation were observed. The cell boundaries of epithelial cells were discernible and the basal membrane integrity was not disrupted. Papillae retained their normal shape. Slight separation and heterogeneity in staining intensity were detected in muscle fibers. It was considered that osmotic changes due to high sugar concentration may contribute to these morphological differences. In this group, the epithelial and stromal structure were well preserved. No significant superiority was observed among the parameters. Parameters were evaluated as 2 and the overall score was calculated as 2.0 (Fig 2).

10% propolis group histological observations

Tissue blocks fixed in 10% propolis were slightly softer (looser) compared than those in the formalin group; however, they still allowed for adequate sectioning with low tissue loss. Microscopic analysis showed mild epithelial shrinkage and increased granular appearance of the cytoplasm within epithelial cells. While the basement

Table 1: Scoring scale of groups.

Group	Epithelium	Nuclear detail	Stromal structure	General score
10% formol	4	4	4	4
10% propolis	2	2	2	2
10% pine honey	2	2	2	2
5% propolis+5% pine honey	2-3	2-3	2-3	2.5

Scoring scale: 0 = Very poor, 1 = Poor, 2 = Fair, 3 = Good, 4 = Excellent.

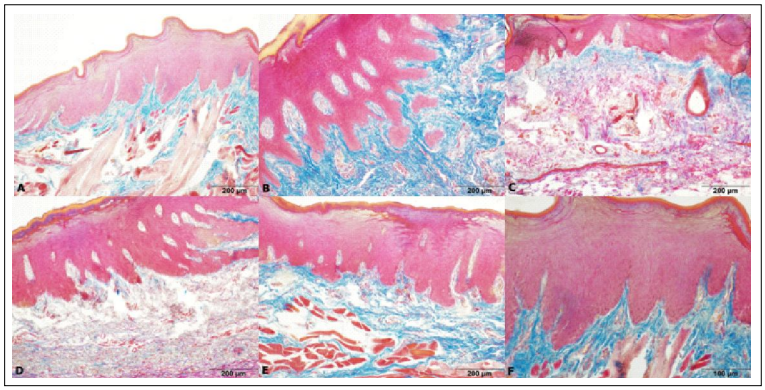


Fig 1: Mallory's triple stained sections of 10% NBF, Apex lingua (A), Corpus lingua (B), Radix lingua (C), Lateral tongue (D), Buccal mucosa (E), Tongue epithelium (F).

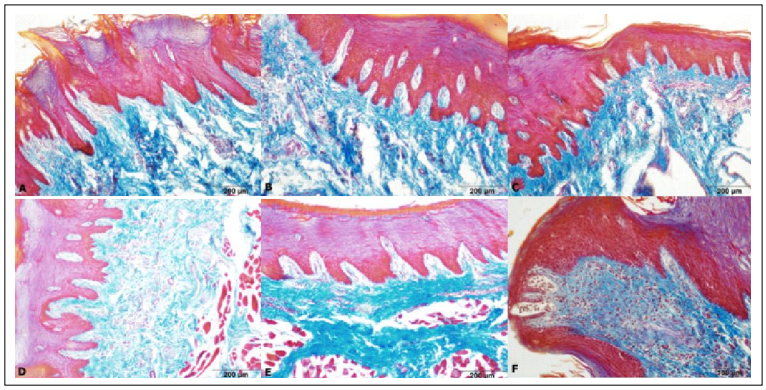


Fig 2: Mallory's triple stained sections of 10% pine honey, Apex lingua (A), Corpus lingua (B), Radix lingua (C), Lateral tongue (D), Buccal mucosa (E), Tongue epithelium (F).

membrane exhibited occasional irregularities and the connective tissue showed more prominent vascularization, the nuclear chromatin distribution remained homogeneous with clear cellular details. Compared to the formalin group, the staining intensity appeared somewhat paler. The skeletal muscle fibers were well-organized with few stromal artifacts. In the 10% propolis group, epithelial integrity was largely preserved, cellular boundaries were distinguishable and nuclear morphology was significantly more regular. The stromal structure showed minimal irregularity. All histological parameters were assessed as moderate, not reaching excellent or good levels. A score of 2 was determined for all parameters and the overall score was calculated as 2.0. These results suggest that propolis provides moderate morphological preservation, likely due to protein denaturation driven by its antimicrobial constituents (Fig 3).

5% propolis + 5% pine honey (Combination group) histological observations

Specimens in the combination group exhibited increased tissue hardness compared to other groups, yet they yielded satisfactory sections. Epithelial preservation was moderate, with localized areas of cellular dissociation; however, cellular borders were distinct and the staining quality was impressive. The integrity of the basement membrane was well-maintained. While the stroma showed slight vacuolization and the muscle fibers exhibited partial disorganization, the overall staining characteristics were superior. In the 5% propolis + 5% pine honey group, partial preservation of epithelial integrity and mild-to-moderate nuclear detail were observed (range 2-3). Overall, this group showed a transition from moderate to good preservation. The average score was calculated as 2.5. Notably, this combination treatment resulted in better morphological outcomes than pine honey alone (Fig 4).

A definitive finding across all groups treated with honey was the absence of identifiable erythrocytes in stained sections of visible erythrocytes in the tongue and buccal tissues following triple staining. This result may be attributed to hydrogen peroxide, a known component of honey. Upon contact, hydrogen peroxide induces cellular alterations including phospholipid reorganization and membrane deformation. It possesses the capacity to form a covalent complex with hemoglobin and spectrin, specific structural proteins of red blood cells. This interaction provides a plausible explanation for the masking or total elimination of erythrocyte staining in these sections.

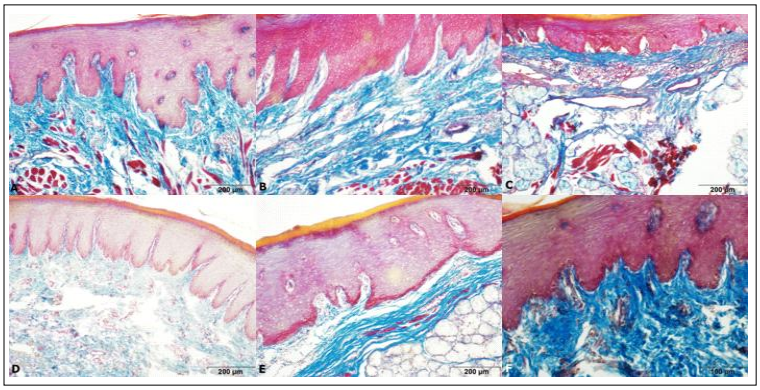


Fig 3: Mallory's triple stained sections of 10% propolis, apex lingua (A), Corpus lingua (B), Radix lingua (C), Lateral tongue (D), Buccal mucosa (E), Tongue epithelium (F).

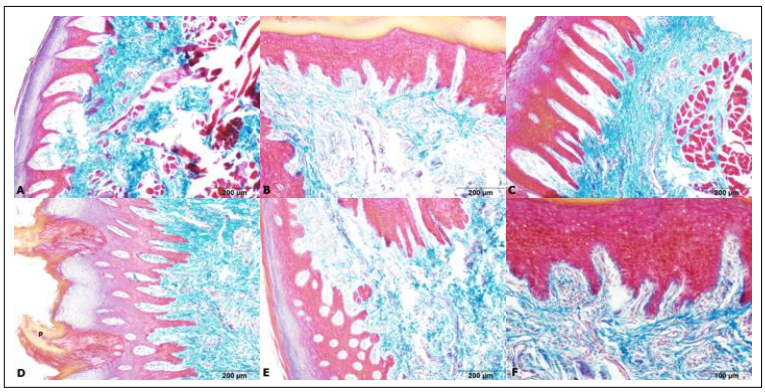


Fig 4: Mallory's triple stained sections of 5% propolis+ 5% pine honey, apex lingua (A), Corpus lingua (B), Radix lingua (C), Lateral tongue (D), Buccal mucosa (E), Tongue epithelium (F).

Despite the variations mentioned, no significant differences were observed between any of the groups regarding the ease of sectioning, ribbon formation, or behavior in the water bath. Furthermore, all fixative groups demonstrated comparable performance in terms of nuclear preservation and staining homogeneity. Comparisons between groups showed the highest damage in the 10% formalin group and the least in the 10% propolis and 10% pine honey groups. Scoring results show that preservation levels increase, especially when natural products are used in combination; however, the maximum score was obtained in the classic formalin group. These findings indicate that natural-based fixatives can preserve the complex morphological architecture of oral tissues at a diagnostically acceptable level.

The fixation of tissue is a foundational step in pathology, intended to preserve the complex interplay of cellular morphology and molecular architecture. While 10% neutral buffered formalin (NBF) has remained the cornerstone of histological processing since the late 19th century, its classification as a Group 1 carcinogen by the IARC has catalyzed a significant paradigm shift toward safer, “green” alternatives. In this study, we investigated the efficacy of propolis and pine honey as natural fixatives, revealing a nuanced performance that balances diagnostic adequacy with unique biochemical artifacts.

Our results demonstrate that while 10% NBF consistently achieved a maximal score (4/4) across all parameters, the natural groups, specifically the propolis-honey combination-yielded a score of 2.5/4. This “good” yet not “perfect” result provides a focal point for debating the practical utility of natural agents. Bugshan *et al*. (2022), argued that propolis could outperform formalin in certain timeframes, a finding that contrasts with our results, where NBF remained superior. This discrepancy may be rooted in the distinct anatomical nature of the goat tongue. The tongue’s high density of skeletal muscle fibers and keratinized stratified squamous epithelium requires rapid and deep penetrative capacity. Formol, through its small molecular size and rapid cross-linking of methylene bridges, excels here. In contrast, the larger phenolic compounds in propolis and the high viscosity of honey likely exhibit slower diffusion rates, leading to the “moderate” scores in stromal and muscular organization observed in our study.

The morphological alterations observed in the honey-fixed groups, specifically the cytoplasmic shrinkage and stromal laxity, warrant a deeper biochemical discussion. Sabarinath *et al*. (2014), provided a crucial quantitative perspective on this by demonstrating that honey fixation results in a significant reduction in cytoplasmic area (CA) and cell perimeter (CP). Our qualitative observations of “granular cytoplasm” and “epithelial shrinkage” align perfectly with their morphometric findings. This phenomenon can be attributed to the high glycemic index and osmolarity of pine honey. The efficacy of honey in preserving dense muscular structures is further corroborated by the work of Piątek-Koziej *et al*. (2019), who investigated honey solutions as an alternative to formaldehyde in cardiac tissue preservation. Their study highlighted that honey provides adequate structural maintenance of myocardial fibers, although it is associated with specific tissue shrinkage patterns-a finding that resonates with the epithelial and stromal alterations observed in our lingual specimens. Given the high protein density and complex fiber orientation shared by both cardiac and lingual muscles, the findings of Piątek-Koziej *et al*. (2019), lend significant weight to our conclusion that honey-based fixation is a viable, non-toxic method for stabilizing specialized muscular tissues. The hypertonic environment created by the honey solution likely induces a sustained osmotic efflux of intracellular water, leading to cellular crenation. However, it is noteworthy that, despite this shrinkage, nuclear detail remained well preserved. This suggests that while honey may alter the volumetric proportions of the cell, it effectively stabilizes the nucleoprotein complex, potentially making it a viable candidate for studies focusing on nuclear grading rather than cytoplasmic volume.

A particularly striking finding in this research, one that distinguishes it from the existing body of literature, is the absolute disappearance of erythrocytes in all honey-treated sections. While Lalwani *et al*. (2015) and Kuriachan *et al*. (2017), noted overall adequate preservation, the specific fate of the vascular components was not extensively detailed in their reports. We propose that this erythrocytic lysis is a direct consequence of the “glucose oxidase” activity inherent in honey, which continuously produces low levels of hydrogen peroxide. This hydrogen peroxide likely interacts with the iron-porphyrin ring of hemoglobin, leading to oxidative damage and the eventual breakdown of the red blood cell membrane. This finding is of paramount importance; it suggests that while honey is an excellent “eco-friendly” alternative for general tissue architecture, as also supported by Patil *et al*. (2015), in their 6-month preservation study it may be unsuitable for diagnosing vascular-rich lesions or identifying hemorrhages in forensic pathology.

Finally, the synergy observed in the combination group (5% Propolis + 5% Honey) suggests that a multi-component natural fixative may be the key to matching formalin’s efficacy. The increased tissue rigidity in this group, though noted as a minor difficulty during sectioning consistent with Al-Maaini and Bryant’s (2006), results, resulted in higher staining quality and sharper cellular boundaries. This indicates that the antimicrobial and protein-precipitating properties of propolis may compensate for the osmotic challenges posed by honey. In conclusion, the results of this study advocate for a balanced view: natural fixatives are not yet a total replacement for formalin in high-stakes diagnostic histopathology, but they offer a robust, non-toxic alternative for educational, research and long-term storage applications where “good” morphological detail is sufficient.

CONCLUSION

In conclusion, the findings of this investigation demonstrate that natural agents specifically propolis and pine honey, offer a viable and bio-friendly alternative for the morphological stabilization of oral tissues. While 10% neutral buffered formalin (NBF) remains the most precise method for detailed histomorphometric analysis, the combination of propolis and honey provides a diagnostically satisfactory level of tissue preservation.

Most notably, this study suggests that propolis holds significant promise as a natural fixative and may serve as an effective interim medium for preserving biopsy specimens prior to formal processing. Our observations align with the perspective that propolis not only maintains structural integrity but may also act as a synergistic agent, enhancing the overall quality of subsequent tissue preservation.

Although the elimination of erythrocytes in honey-based groups and the presence of minor osmotic shrinkage are notable characteristics, the non-toxic and cost-effective nature of these compounds advocates for their integration into routine laboratory workflows, particularly in settings where carcinogen exposure must be minimized. Ultimately, these natural solutions represent a robust step toward "Green Histopathology" providing a sustainable balance between diagnostic efficacy and laboratory safety.

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Disclaimers

The views and conclusions expressed in this article are solely those of the author and do not necessarily represent the views of their affiliated institutions. The author are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

This study used material from a slaughterhouse and the tissue samples were not subject to ethical committee approval in accordance with the Regulation on the Working Procedures and Principles of Animal Experimentation Ethics Committees of the Ministry of Forestry and Water Affairs of the Republic of Turkey (dated 15 February 2014, No. 28914).

Conflict of interest

The author declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish or preparation of the manuscript.

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