## Original Article

# Natural stain (*Kumkum*) formulated by the extract of *Curcuma aromatica* and slaked lime in histostaining of oral tissues: An observational study

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## Abstract

**Background:** The pharmacological actions of *Curcuma aromatica* (wild turmeric) such as anti-inflammatory, antitumor, antifungal, antimicrobial and wound healing have been recognized since ages. However, its role as a natural histological stain has not been explored till date.

**Aim:** To evaluate the efficacy of natural substance-*Kumkum* prepared from the extract of *C. aromatica* and slaked lime in staining the biopsied oral tissues

**Materials and Methods:** A cohort study that used 60 formalin fixed paraffin embedded soft and hard tissue specimens from institutional archives were subjected to sectioning and stained using *Kumkum* and hematoxylin and eosin (H and E). The slides were evaluated for their staining efficacy and results were statistically analyzed using Wilcoxon signed-rank test and Independent 't' test.

**Results:** The mean of the overall parameters assessed for staining efficacy did not show statistically significant difference between the study groups in normal and pathological specimens for tooth ($P = 0.410$ and $0.484$), bone ($P = 0.133$ and $0.157$) and soft tissues ($P = 0.186$ and $0.113$), respectively. This suggests that *Kumkum* staining efficacy is equivalent to that of routine H and E for oral tissues. Structures such as dentinoenamel junction, dentinal tubules, incremental lines of cementum, reversal and resting lines, osteocytic canaliculi, mature and immature bone could be appreciated better in *Kumkum* stained slides, thereby rendering a special staining property to *Kumkum* stain.

**Conclusion:** To our knowledge, this study is the first of its kind to have used *Kumkum* stain obtained from *C. aromatica* for the differentiation of the components of tooth, bone and soft tissue structures in histostaining of oral tissues. The naturally prepared *Kumkum* stain possesses dual staining property both in routine and differential staining. This facilitates diagnosis of fibro-osseous lesions, bony, collagen and muscular pathologies. The natural stain also finds application in forensic odontology for age estimation.

**Keywords:** Coloring agents, *Curcuma aromatica*, hematoxylin and eosin and histostaining

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## INTRODUCTION

Synthetic dyes that are commonly employed in histopathology for staining tissue sections are harmful to the laboratory personnel and cause skin allergies, respiratory tract infections, irritation and various types of cancers due to the production of toxic waste products on prolonged exposure. For example, dyes with azo bonds, nitro- or amino groups are tumorigenic in causing hepatic and renal carcinomas. To prevent the harmful effects of synthetic dyes, there is a need to identify certain natural substances possessing staining properties, yet are biocompatible, biodegradable and eco-friendly. Therefore, natural dyes have gained interest in the recent years and substantial research is in progress to replace the synthetic dyes.[1]

Natural dyes are mainly prepared from primary sources such as fruits, flowers, leaves, roots and barks of plants and trees. These natural compounds have been used in a variety of instances from tribal tattoos and religious or cultural customs to various activities such as painting, decorative art and decoration of clothes. In the medical field, these stains are commonly used in anatomical, surgical and histopathology for the diagnosis of various diseases and to locate the tumor-free margins. The stains aid in the microscopic examination of cells, nucleic acids and proteins. The bonds between the stain and tissue substrates are mainly due to acid base reactions. Since every tissue is made of multiple structures, their staining properties also vary mandating the use of a combination of stains to prepare tissue sections of diagnostic quality.[2]

Recently, researchers have examined the potential use of natural substances such as curcumin, beetroot, ginger, *Pterocarpus osun*, rose, henna and *Hibiscus sabdariffa* in staining tissues and microbes. *Kumkum* is one such substance that imparts red color to the tissues and is prepared by mixing turmeric and slaked lime.

*Kumkum* powder can be prepared by both natural and commercial methods. Naturally, *Kumkum* is prepared from mixing of turmeric with lime water and camphor or from saffron or combination of turmeric and slaked lime. Commercially, it is prepared by the combination of azo dyes, corn starch, fragrances, chalk powder, ground-nut oil, tragacanth gum, turmeric powder and parabens. However, literature review reveals that the *Kumkum* obtained by the combination of *Curcuma aromatica* and slaked lime as a natural substance in histostaining of oral tissues is rarely employed. Hence, there is a need to assess the efficacy of *Kumkum* prepared by the amalgamation of *C. aromatica* and slaked lime in histostaining of oral tissues.[3]

## MATERIALS AND METHODS

It was a cohort study that utilized 60 formalin fixed paraffin embedded (FFPE) archival tissues (normal - 30 and pathological - 30 soft and hard tissues) from the Department of Oral Pathology and Microbiology, Faculty of Dental Sciences, Ramaiah University of Applied Sciences (RUAS). The study was approved by the University Ethics committee for Human Trials of our institutional review board (UECHT/2016-18/PGDT). The study was exempted of informed consent. The histopathologically confirmed different cases of normal oral mucosa and bone (10 each), soft and hard tissue pathologies (10 each), normal and carious decalcified tooth specimens (10 each) were included in the study. Biopsies revealing inadequate tissue structures on histopathology were excluded from the study.

Rhizomes of *C. aromatica* were collected, cut into small pieces and boiled at 95°C. The cut pieces were dried and milled to form fine powder using household mixer-grinder. Five milligrams of this turmeric powder, weighed by an electronic weighing machine was mixed with 2.5 mg of slaked lime. 0.02 mg of sodium bicarbonate (mordant) and 20 ml of 80% ethanol were added to the above mixture. The staining intensity of the extract was enhanced by incorporation of 0.05 mg of jaggery. The final product was stirred well and filtered twice using the Whatman number 1 filter paper.

Two sets of 4 μm thick sections from the selected FFPE tissue blocks were obtained. The slides were deparaffinized in xylene and rehydrated using descending grades of alcohol. The first set of slides was subjected to routine hematoxylin and eosin (H and E) staining using the standard protocol.[4] The second set of slides was stained with the prepared *Kumkum* stain. The staining protocol for *Kumkum* stain has been shown in Figure 1.

The stained slides were evaluated independently by 2 oral pathologists using binocular microscope (Olympus CX21FS1) employing a criterion that was specifically devised for this study. The epithelium and connective tissue components of normal and pathological soft tissues were evaluated for the parameters – homogeneity, staining intensity, nuclear and cytoplasmic staining. For the evaluation of bone and tooth specimens, the parameters considered were homogeneity, staining intensity, specificity and differentiation. The slides were evaluated for all the above parameters semi-quantitatively with scores of 0, 1, 2, 3 for negative, poor, good and excellent staining, respectively, that was modified by the criteria of Kumar *et al.*[5]
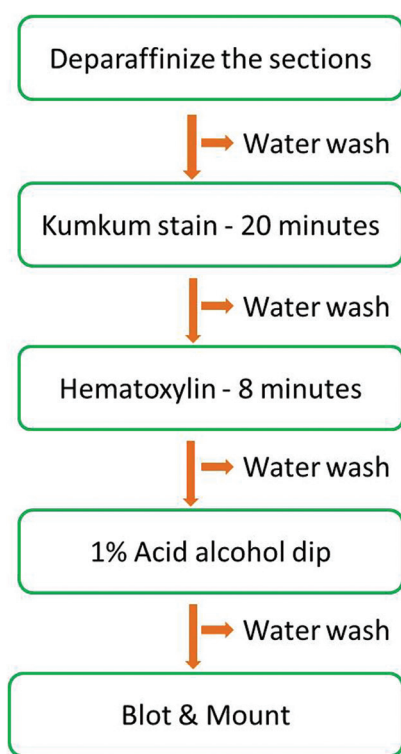
**Figure 1:** Flow chart showing the staining procedure for *kumkum* stain

The comparison of staining efficacy between *Kumkum* and H and E stained sections in normal and pathological soft and hard tissues was performed using Wilcoxon sign rank test. The overall mean of the two study groups was evaluated using independent *t*-test. $P < 0.05$ was considered statistically significant. The tests were carried out using Statistical Package for the Social Sciences for Windows, Version 22.0 (2013) (IBM Corp., Armonk, NY, USA).

## RESULTS

Evaluation of various parameters for staining efficacy of *Kumkum* and H and E on tooth, bone and soft tissue sections revealed the following findings as shown in Tables 1-3.

## DISCUSSION

*Kumkum* is a compound that is commercially available or naturally prepared using turmeric and basic components. Turmeric that forms the bulk of *Kumkum* preparation, has been used for centuries in many Eastern countries both as a spice and as a medicine. Turmeric has been used from 4000 years by Vedic culture in India.

Scientifically termed as "Curcuma" and well known as "Turmeric", it belongs to "Zingiberaceae" family. There are mainly two types of turmeric namely Curcuma longa and

*C. aromatica*. Turmeric is obtained from a perennial, erect and pointed leafy plant with funnel shaped yellow flowers. The rootstalk of the plant called rhizome is cleaned, boiled and dried to produce yellow-colored powder, turmeric.[6,7]

Chemically, curcumin consists of two aromatic units each having-O-methoxy and phenolic substituents linked by "polyene" within which either keto-enol or di-keto moieties are present. Thus, curcumin is present as anionic or electrically neutral species. The solubility of turmeric is poor in water and acidic solutions, but highly soluble in organic solvents and alkaline solutions.[8] The principle of staining is based on acid–base reaction as depicted in the Figure 2.

H and E staining has been an age old technique in tissue based diagnosis. They have been able to delineate differences in tissue type and cellular components using the inherent uptake properties of various tissues. When certain structures display similar staining characteristics in H and E, special staining techniques are of great value. These special stains are of diagnostic utility in everyday practice and may be used to differentiate various types of connective tissue components such as cells, cartilage, bone, collagen, blood, muscle, elastic fibers, etc., to determine the cell of origin of the tumor.[1]

Tooth: In the present study, 10 samples each of normal and pathological (carious) decalcified tooth sections that were stained with *Kumkum* were evaluated for homogeneity, staining intensity and differentiation in staining. All these parameters in both the study groups of normal and carious teeth showed similar staining characteristics with statistically insignificant 'p' values of 0.41 and $P = 0.48$, respectively, Table 3.

Decalcified sections of *Kumkum* stained tooth sections imparted orange color to the dentin and dark red color to dentinoenamel junction (DEJ) and incremental lines of cementum. DEJ, dentinal tubules, cementocytes and their canaliculi were better appreciated in *Kumkum* stained slides as compared to H and E [Figure 3 and Table 1]. However, it was not statistically significant suggesting that the staining efficacy of *Kumkum* was similar to that of H and E. As structures such as incremental lines of Salter and dentin showed high contrast, *Kumkum* staining may find application as a differential stain in histopathology. Literature search has not revealed studies favoring the results of present study. However, certain synthetic compounds have been used as special stains for cementum such as picro-thionin for dentin, modified Gallego's, Toluidine blue and Alcian blue.

**Table 1: Comparison of homogeneity, staining intensity and differentiation of dentinal tubules, dentinoenamel junction and incremental lines of cementum in normal and pathological teeth in Hematoxylin and Eosin and kumkum stained sections**

| Dentinal tubules | Type of specimen | Homogeneity | | | | Staining intensity | | | | Differentiation | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Negative | Poor | Good | Excellent | Negative | Poor | Good | Excellent | Negative | Poor | Good | Excellent |
| H and E | Normal tooth | 0 | 0 | 2 | 8 | 0 | 0 | 2 | 8 | 0 | 0 | 3 | 7 |
| | Pathological tooth | 0 | 0 | 2 | 8 | 0 | 0 | 3 | 7 | 0 | 0 | 1 | 9 |
| Kumkum stain | Normal tooth | 0 | 0 | 1 | 9 | 0 | 0 | 2 | 8 | 0 | 0 | 2 | 8 |
| | Pathological tooth | 0 | 0 | 2 | 8 | 0 | 0 | 3 | 7 | 0 | 0 | 1 | 9 |
| *Z* scores | Normal tooth | −1.000 | | | | 0.000 | | | | −0.447 | | | |
| | Pathological Tooth | −1.000 | | | | 0.000 | | | | 0.000 | | | |
| *P* (two-tailed) | Normal tooth | 0.317 | | | | 1.000 | | | | 0.655 | | | |
| | Pathological Tooth | 0.317 | | | | 1.000 | | | | 1.000 | | | |
| **Dentinoenamel Junction** | **Type of specimen** | **Homogeneity** | | | | **Staining intensity** | | | | **Differentiation** | | | |
| | | Negative | Poor | Good | Excellent | Negative | Poor | Good | Excellent | Negative | Poor | Good | Excellent |
| H and E | Normal tooth | 0 | 0 | 3 | 7 | 0 | 0 | 1 | 9 | 0 | 0 | 3 | 7 |
| | Pathological tooth | 0 | 0 | 0 | 4 | 0 | 0 | 7 | 3 | 0 | 0 | 4 | 6 |
| Kumkum stain | Normal tooth | 0 | 0 | 0 | 10 | 0 | 0 | 1 | 9 | 0 | 0 | 2 | 8 |
| | Pathological tooth | 0 | 0 | 1 | 5 | 4 | 0 | 0 | 10 | 0 | 1 | 5 | 4 |
| *Z* scores | Normal tooth | −1.732 | | | | 0.000 | | | | −.447 | | | |
| | Pathological tooth | −2.000 | | | | −2.646 | | | | −1.134 | | | |
| *P* (two-tailed) | Normal tooth | 0.083 | | | | 1.000 | | | | 0.655 | | | |
| | Pathological tooth | 0.046+ | | | | 0.008 | | | | 0.257 | | | |
| **Incremental lines of cementum** | **Type of specimen** | **Homogeneity** | | | | **Staining intensity** | | | | **Differentiation** | | | |
| | | Negative | Poor | Good | Excellent | Negative | Poor | Good | Excellent | Negative | Poor | Good | Excellent |
| H and E | Normal tooth | 0 | 0 | 4 | 6 | 0 | 3 | 7 | 0 | 2 | 8 | 0 | 0 |
| | Pathological tooth | 0 | 0 | 1 | 9 | 0 | 3 | 7 | 0 | 0 | 10 | 0 | 0 |
| Kumkum stain | Normal tooth | 0 | 0 | 2 | 8 | 0 | 0 | 3 | 7 | 0 | 0 | 0 | 10 |
| | Pathological tooth | 0 | 0 | 1 | 9 | 0 | 0 | 0 | 10 | 0 | 0 | 0 | 10 |
| *Z* scores | Normal tooth | −1.000 | | | | −2.640 | | | | −2.972 | | | |
| | Pathological tooth | 0.000 | | | | −2.889 | | | | −3.162 | | | |
| *P* (two-tailed) | Normal tooth | 0.317 | | | | 0.008 | | | | 0.003 | | | |
| | Pathological tooth | 1.000 | | | | 0.004 | | | | 0.002 | | | |

Mudhiraj *et al*. used modified Gallego's stain to differentiate various hard tissue structures of ground and decalcified sections of teeth in normal and pathological lesions of the oral cavity. They found that enamel appears pink and cementum stains red whereas dentin and bone stained green color in normal and pathological lesions.[9] Similarly, Shukla *et al*. proposed a study using various stains such as cresyl violet, H and E, toluidine blue and periodic acid Schiff for cementum in different cemento-osseous lesions under light and florescence microscopes and revealed that cresyl violet showed better contrast than all stains in both decalcified and ground sections and therefore is of diagnostic value in bone pathologies.[10]

Sarbeen and Jayaraj aimed to characterize the composition of cementum based on its staining affinity for various stains such as H and E, toluidine blue and combination of Alcian blue–nuclear fast red stains. They found that in toluidine blue staining, cementum showed deep blue color in contrast to dentin that was pale blue, whereas Alcian blue stain with nuclear fast red counterstaining revealed pink areas of dentin and cementum. The authors concluded that differentiation of cementum is necessary for developing therapeutic approach for cemental regeneration.[11]

To our knowledge, studies with natural substances used for distinguishing different tooth structures are rarest of rare. Hence, the present study is the first of its kind to characterize the different structures of tooth using naturally prepared stain *Kumkum* (*C. aromatica* and slaked lime).

**Bone**

In the present study, 10 samples each of normal and pathological decalcified bone sections stained with *Kumkum* (*C. aromatica* and slaked lime) were compared with routine H and E. The parameters that were assessed for staining efficacy of bone were similar to that applied for the evaluation of teeth. Comparison of all the parameters of staining between the study groups showed statistically insignificant difference for both normal and pathological bone tissues with $P = 0.133$ and 0.157, respectively, as shown in the Table 3. However, *Kumkum* (*C. aromatica* and slaked lime) showed better specificity and staining intensity for normal and pathological bone than routine H and E.

Decalcified sections of bone in *Kumkum* stain showed dark red color for immature bone and light orange-red for mature bone with better differentiation. Canaliculi of osteocytes, reversal and resting lines in bone showed superior staining properties than routine H and E

**Table 2: Comparison of homogeneity, staining intensity, specificity and differentiation of immature bone, reversal and resting lines and canaliculi in normal and pathological bone in Hematoxylin and Eosin and kumkum stained sections**

| Immature bone | Type of specimen | Homogeneity | | | | Staining intensity | | | | Specificity | | | | Differentiation | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Negative | Poor | Good | Excellent | Negative | Poor | Good | Excellent | Negative | Poor | Good | Excellent | Negative | Poor | Good | Excellent |
| H and E | Normal bone | 0 | 0 | 0 | 10 | 0 | 0 | 3 | 7 | 0 | 0 | 4 | 6 | 0 | 8 | 2 | 0 |
| | Pathological bone | 0 | 0 | 7 | 3 | 0 | 0 | 8 | 2 | 0 | 0 | 10 | 0 | 0 | 0 | 2 | 8 |
| Kumkum stain | Normal bone | 0 | 0 | 1 | 9 | 0 | 0 | 0 | 10 | 0 | 0 | 0 | 10 | 0 | 0 | 0 | 10 |
| | Pathological bone | 0 | 0 | 0 | 10 | 0 | 0 | 3 | 7 | 0 | 0 | 3 | 7 | 0 | 0 | 3 | 7 |
| Z scores | Normal bone | −1.000 | | | | −2.919 | | | | −2.000 | | | | −2.972 | | | |
| | Pathological bone | −2.646 | | | | −2.236 | | | | −2.646 | | | | −2.879 | | | |
| P (two-tailed) | Normal bone | 0.317 | | | | 0.004 | | | | 0.046 | | | | 0.003 | | | |
| | Pathological bone | 0.008 | | | | 0.025 | | | | 0.008 | | | | 0.004 | | | |

| Reversal and Resting line | Type of specimen | Homogeneity | | | | Staining intensity | | | | Specificity | | | | Differentiation | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Negative | Poor | Good | Excellent | Negative | Poor | Good | Excellent | Negative | Poor | Good | Excellent | Negative | Poor | Good | Excellent |
| H and E | Normal bone | 0 | 0 | 4 | 6 | 0 | 0 | 8 | 2 | 0 | 0 | 1 | 9 | 7 | 3 | 0 | 0 |
| | Pathological bone | 0 | 0 | 7 | 3 | 0 | 7 | 3 | 0 | 4 | 6 | 0 | 0 | 8 | 2 | 0 | 0 |
| Kumkum stain | Normal bone | 0 | 0 | 0 | 10 | 0 | 0 | 0 | 10 | 0 | 0 | 2 | 8 | 0 | 0 | 0 | 10 |
| | Pathological bone | 0 | 0 | 0 | 10 | 0 | 0 | 0 | 10 | 0 | 0 | 0 | 10 | 0 | 0 | 0 | 10 |
| Z scores | Normal bone | −2.000 | | | | −2.828 | | | | −2.913 | | | | −2.919 | | | |
| | Pathological bone | −2.646 | | | | −2.889 | | | | −2.889 | | | | −2.972 | | | |
| P (two-tailed) | Normal bone | 0.046 | | | | 0.005 | | | | 0.004 | | | | 0.004 | | | |
| | Pathological bone | 0.008 | | | | 0.004 | | | | 0.004 | | | | 0.003 | | | |

| Canaliculi | Type of specimen | Homogeneity | | | | Staining intensity | | | | Specificity | | | | Differentiation | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Negative | Poor | Good | Excellent | Negative | Poor | Good | Excellent | Negative | Poor | Good | Excellent | Negative | Poor | Good | Excellent |
| H and E | Normal tooth | 0 | 1 | 4 | 5 | 0 | 2 | 8 | 0 | 7 | 3 | 0 | 0 | 7 | 3 | 0 | 0 |
| | Pathological tooth | 0 | 1 | 7 | 2 | 1 | 9 | 0 | 0 | 9 | 1 | 0 | 0 | 9 | 1 | 0 | 0 |
| Kumkum stain | Normal tooth | 0 | 0 | 2 | 8 | 0 | 0 | 1 | 9 | 0 | 0 | 0 | 10 | 0 | 0 | 1 | 9 |
| | Pathological tooth | 0 | 0 | 2 | 8 | 0 | 0 | 0 | 10 | 0 | 0 | 3 | 7 | 0 | 0 | 0 | 10 |
| Z scores | Normal tooth | −1.414 | | | | −2.887 | | | | −2.919 | | | | −2.889 | | | |
| | Pathological tooth | −2.646 | | | | −3.051 | | | | −2.889 | | | | −3.051 | | | |
| P (two-tailed) | Normal tooth | 0.157 | | | | 0.004 | | | | 0.004 | | | | 0.004 | | | |
| | Pathological tooth | 0.008 | | | | 0.002 | | | | 0.004 | | | | 0.002 | | | |

| Various pathologies | Type of specimen | Homogeneity | | | | Staining intensity | | | | Specificity | | | | Differentiation | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Negative | Poor | Good | Excellent | Negative | Poor | Good | Excellent | Negative | Poor | Good | Excellent | Negative | Poor | Good | Excellent |
| H and E | Pathological tooth | 0 | 0 | 3 | 7 | 0 | 0 | 6 | 4 | 0 | 0 | 3 | 7 | 0 | 0 | 6 | 4 |
| Kumkum stain | Pathological tooth | 0 | 0 | 0 | 10 | 0 | 0 | 0 | 10 | 0 | 0 | 0 | 10 | 0 | 0 | 0 | 10 |
| Z scores | Pathological tooth | −2.919 | | | | −2.000 | | | | −2.919 | | | | −2.000 | | | |
| P (two-tailed) | Pathological tooth | 0.004 | | | | 0.046 | | | | 0.004 | | | | 0.046 | | | |

H and E: Hematoxylin and Eosin

staining [Figure 4 and Table 2]. Literature search did not reveal studies using natural stains for visualizing bone. However, various synthetic special stains such as modified Gallego's (MGS), verdeluz orange G-acid fuchsin (VOF) and methylene blue-acid fuchsin (MB-AF) have been used till date for the demonstration of bone.

Putns and Desa (1977) employed modified Attwood's stain to characterize decalcified bone sections and concluded that



**Figure 2:** Principle of naturally prepared *kumkum* stain on hard and soft tissues

it can be used as a differential stain to distinguish woven bone from lamellar bone.[12] In 2012, Gupta *et al.* performed a study using MB-AF staining technique to differentiate the organic matrix of osteoid from hyalinized stroma and revealed that the MB-AF staining technique showed faint pink color for osteoid with blue stromal background. The authors concluded that MB-AF is a simple, single step procedure, but it cannot be used as a specific stain for types of calcification other than bone and osteoid.[13]

Belaldavar *et al.* (2014) assessed the efficacy of the VOF stain for differentiating hard and soft connective tissue components and compared them with H and E. They found that VOF demonstrated greater staining intensity with good contrast for hard tissues than H and E. Therefore, they concluded that VOF stain is rapid, easy and single step staining technique which helps in characterizing the maturity of tissues.[14] In 2017, Kunche *et al.* used MGS and VOF stains to differentiate various hard tissue components and found that VOF stained purple red color for bone tissues, red for cementum and blue for collagen

**Table 3: Overall mean of the parameters used to evaluate the staining efficacy of normal and pathological tooth, bone and soft tissue specimens**

| Tissue | Mean±SD for H and E stain | Mean±SD for Kumkum stain | P |
|---|---|---|---|
| Normal tooth | 39±2.667 | 50±2.234 | 0.410 |
| Pathological tooth | 50±2.079 | 58±1.912 | 0.484 |
| Normal bone | 37±2.821 | 57±1.578 | 0.133 |
| Pathological bone | 45±2.044 | 68±1.252 | 0.157 |
| Normal soft tissue | 101±1.509 | 94±1.160 | 0.186 |
| Pathological soft tissues | 111±1.829 | 107±1.252 | 0.113 |

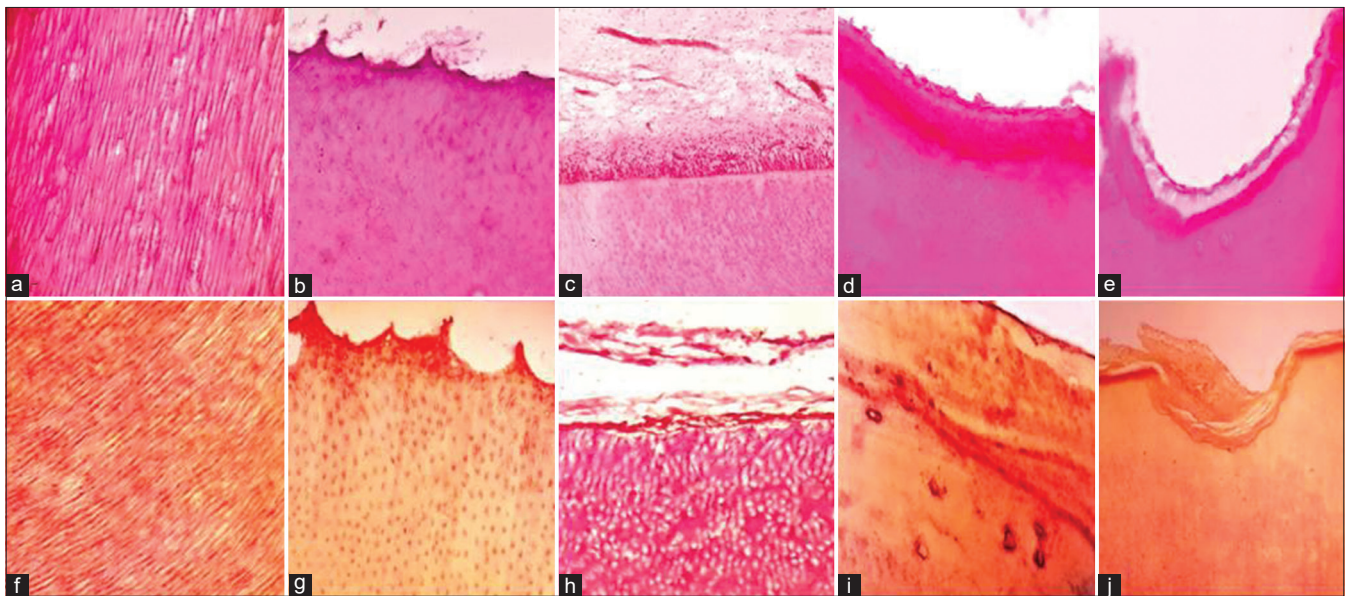SD: Standard deviation, H and E: Hematoxylin and Eosin



**Figure 3:** Photomicrographs of hematoxylin and eosin and *kumkum* stained normal and pathological tooth specimens (×400): (a and f) Dentinal tubules, (b and g) dentinoenamel junction, (c and h) layers of pulp (×100), (d and i) cementum, (e and j) dental caries

whereas MGS stained bone green-blue, cementum with red and collagen with blue colors. The authors confirmed that VOF showed better staining intensity for hard tissues and exhibited good contrast with surrounding connective tissue.[15] In the present study a natural compound, *Kumkum* was found to differentiate immature from mature bone. Structures such as canaliculi of osteocytes, resting and reversal lines of bone had a better contrast with increased staining intensity. Hence, *Kumkum* may be used as a differential stain for bone in diagnosing bone pathologies and also in assessing the phase of bone remodeling.

Soft tissues: 10 samples each of normal and pathological soft tissue sections were stained with *Kumkum* (*C. aromatica* and slaked lime) and compared with routine H and E staining. There was no statistically significant difference in normal ($P = 0.186$) and pathological ($P = 0.113$) soft tissues between the study groups [Table 3]. This infers that *Kumkum* stain showed similar staining efficacy to that of routine H and E. *Kumkum* imparted varying shades of red color to different structural components in tissue sections. The cellular cytoplasm and nuclei showed light red and brick red colors, respectively. The connective tissue constituents such as collagen, muscle and nerve fibers appeared brick red whereas red blood cells presented with yellow color [Figure 5].

There are numerous studies available in the literature that has employed natural and synthetic stains for the demonstration of various soft tissue structures. Natural substances such as *Pterocarpus osun, Curcuma longa, Hibiscus sabdariffa, Ceratonia Siliqua bark, Zingiber officinale* and henna have been used for staining collagen and muscle fibers and black mulberry for staining nerve fibers in human tissues. On the other hand, several synthetic special stains are also available to stain these structures.[16-21]

Adisa *et al*. studied the use of henna leaves extract as a natural cytoplasmic stain in liver tissues and found that hepatocytes stained golden brown color with well-defined cytoplasmic boundary and concluded that henna leaves extract could be a suitable cytoplasmic stain in histopathology.[16] Similarly, in 2015, Ajileye *et al*. conducted a study to explore the staining potential of *Zingiber officinale* (ginger) extract on tissue sections and found that nuclei stained deeply green color whereas the cytoplasm and muscle fibers showed yellow color. The authors concluded that extract of *Zingiber officinale* can be a promising natural histological dye for cytoplasm of the cell and muscle fibers.[17]

Kumar *et al*. used Curcuma longa extract to identify its staining ability on human tissue sections in comparison to the routine eosin dye and found that deep yellowish orange color was appreciated in collagen and muscle fibers. The authors suggested that turmeric could be a good natural histological dye which may authenticate its role in the treatment of collagen and muscle disorders.[5]

Gupta *et al*. (2014) investigated the staining ability of prepared stain Kumkum on microbes and found that Kumkum imparted light red hue to the Escherichia coli. They concluded that Kumkum stain is safer and can be used to stain microbes.[22] Black mulberry extract was used by Tousson and Al-Behbehani to explore its staining efficacy on nerve tissues and appreciated light brown shade in neurons and faint staining in the astrocytes of thalamus and hypothalamus. The authors concluded that black mulberry extract could be a promising natural dye for neurons and astrocytes in brain tissues.[23]

Several synthetic special stains are also used in the diagnosis of pathological diseases. The special stains are Van Gieson, Masson trichrome, Periodic Acid Schiff, Alcian blue, Picrosirus red, etc., Most of these stains are available as commercial preparations which are rapid, easy to use with less time consumption. In 2015, Sharma *et al*. carried out a study to evaluate the nature of collagen in oral epithelial dysplasia (OED) and oral squamous cell carcinoma (OSCC) using Picrosirius Red Stain (PSR) under polarizing microscopy. In OED, PSR staining showed yellowish-orange to greenish yellow birefringence with the advancement of grade. Similarly, in OSCC, the birefringence ranged from reddish/yellowish orange to greenish-yellow, suggesting a transition from mature to immature collagen. The authors concluded that PSR stain could be useful in identifying the stromal changes in OED and OSCC.[24]

Yasseen studied the staining efficacy of Cresyl Violet and Toluidine blue stains in the detection of ganglion cells in suspected Hirschsprung's disease. He found that in Hirschsprung's disease, Cresyl violet and Toluidine blue stains were superior to H and E and suggested that these stains play an important role in the detection of ganglion cells in Hirschsprung's disease.[25] Calvi *et al*. in 2012 examined collagen in skeletal muscle using various special stains such as Picrosirius red, Masson's trichrome, reticulin and immunostained with different antibodies for collagen Types I, II, III, IV and V. The authors concluded that Masson's trichrome and Picrosirus red stains provided superior staining properties and helped in assessing the qualitative and quantitative influence of collagen in muscular diseases.[26]
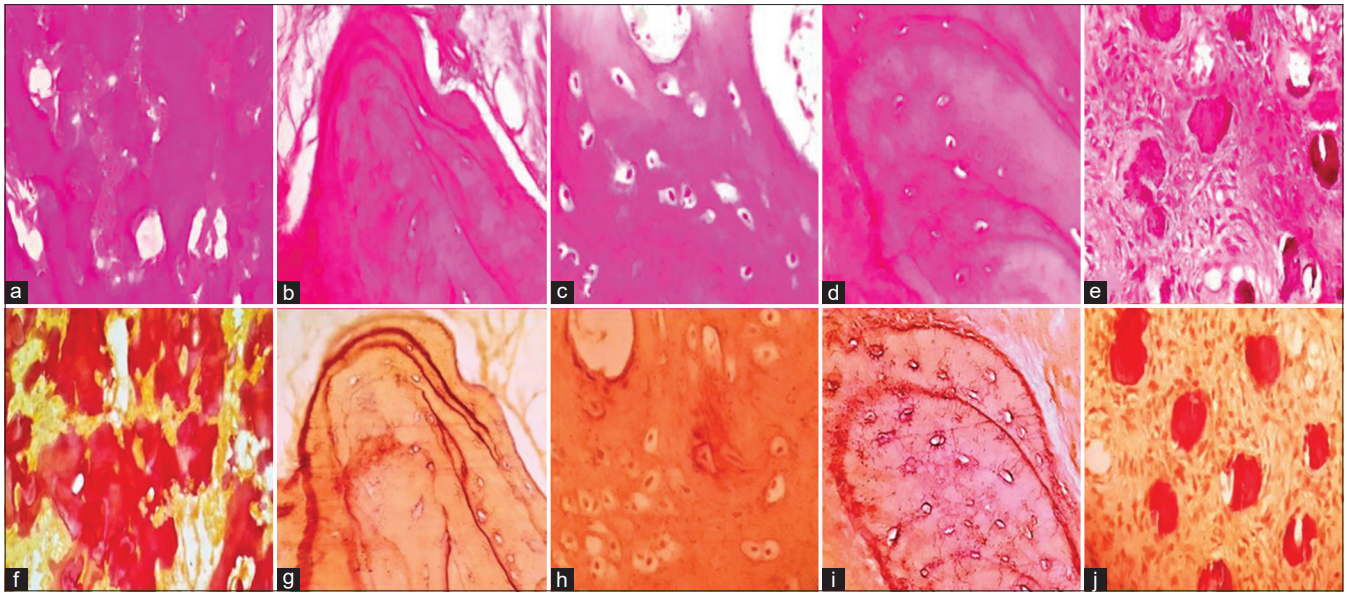
**Figure 4:** Photomicrographs of hematoxylin and eosin and *kumkum* stained normal and pathological bone tissues: (a and f) Bone (×100), (b and g) reversal and resting lines, (c and h) Cells of bone, (d and i) lacunae and canaliculi, (e and j) bone pathology (×400)
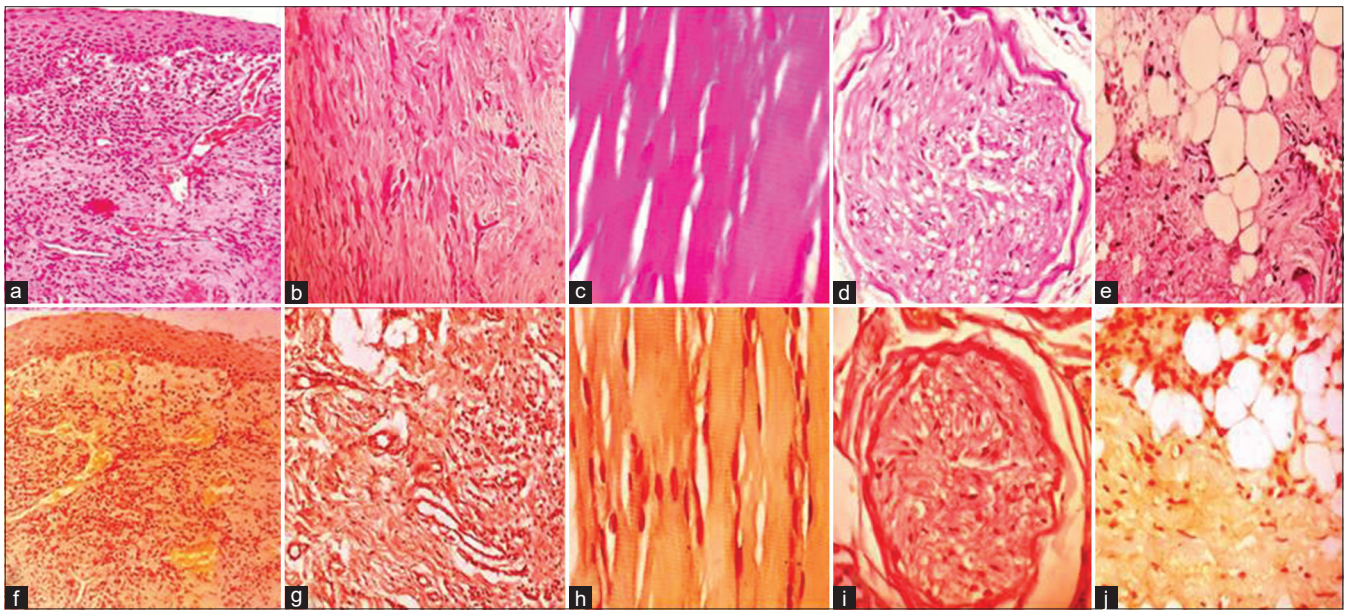


**Figure 5:** Photomicrographs of hematoxylin and eosin and *kumkum* stained normal mucosa and pathological soft tissues: (a and f) Epithelium (×100), (b and g) collagen fibers, (c and h) muscle fibers, (d and i) nerve, (e and j) adipose tissue (×400)

The staining procedure using *Kumkum* is technique sensitive as inappropriate proportions (2:1) of *C. aromatica* and slaked lime may have adverse effects on the results. As the shelf life of the stain has not been evaluated in the present study, the longevity of the stain is questionable. Staining procedures were carried out at room temperature; therefore, the effect of variation in temperature on the staining ability of *Kumkum* is not known.

Further studies may be carried out to evaluate the staining efficacy of *Kumkum* prepared using alternate methods that employs lime water in combination with *C. aromatica*. The shelf

life of the stain at different time intervals with larger sample size may be investigated to determine its prolonged use. Diagnostic role as a special stain in histopathology for fibro-osseous lesions need to explored. Also, further studies required to compare the efficacy of *Kumkum* stained tooth sections with that of routine H and E for age estimation in forensic investigation.

## CONCLUSION

It is noteworthy to conclude that *Kumkum* prepared from the extract of *C. aromatica* and slaked lime could be a safer

alternative natural stain that has shown similar staining characteristics as compared to H and E. Staining using *Kumkum* is cost effective, eco-friendly, non-allergic and noncarcinogenic with easy availability.

*Kumkum* has a dual property of both nuclear and cytoplasmic staining ability in histopathology for various soft and hard tissues. Decalcified tooth sections with *Kumkum* stain showed different shades of color for different mineralized structures. However, the incremental lines of Salter had a better contrast in *Kumkum* staining, hence it may find application in forensic sciences for age estimation.

Decalcified bone sections with *Kumkum* stain helped in differentiating immature from mature bone. There was a clear distinction between hyalinized stroma and calcifications in *Kumkum* stained sections. Structures such as canaliculi of osteocytes and reversal and resting lines in *Kumkum* stain showed superior staining properties. Hence, *Kumkum* may be used as a natural stain for studying the normal bone structures, as a differential stain in diagnosing bone pathologies such as fibro-osseous lesions, neoplasms and also in assessing the phases of bone remodeling.

In soft tissue sections stained using *Kumkum*, connective tissue structures such as collagen, muscle fibers and red blood cells showed better contrast and therefore finds application in collagen and muscular disorders. Hence, *Kumkum* may be used as a natural dye for routine and differential staining of oral tissues in histopathology.

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### Conflicts of interest
There are no conflicts of interest.

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