

Staining Efficacy of Rose Extract in Comparison with Eosin Stain: A Histological Study on Oral Tissues

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ABSTRACT

Introduction: Natural extract dyes are eco-friendly and non-hazardous when compared with synthetic stains. Routinely, hematoxylin and eosin (H&E) stain is used to stain tissues. Eosin is a synthetic stain and attempts have been made to substitute eosin with a natural dye, one among them being rose extract. Staining using rose extract is economical, easily available and has not been explored before.

Aims and objectives: The study aims to compare between two different extraction methods for rose, namely maceration and soxhlet, to compare the staining efficacy of rose with (REM) and without mordant, to compare the efficacy of rose extracts with synthetic eosin, in both normal and pathological oral tissues [e.g., oral squamous cell carcinoma (OSCC)].

Materials and methods: Rose was dried, powdered, and extracted by maceration and soxhlet techniques. Tissue sections were stained with and without the mordant potassium alum. Hematoxylin and rose (H&R) and H&E stained normal and pathologic tissues were compared. Statistical analysis was done using Chi-square test.

Results: Statistically significant results were observed in sections stained by soxhlet method than that of maceration, sections stained by REM was comparatively better than those stained with rose extract alone, the normal and pathologic tissues stained with H&R gave comparable result with H&E.

Conclusion: The REM can be used as a substitute to eosin for the staining of tissues.

Clinical significance: Natural substitute for synthetic eosin.

Keywords: Hematoxylin, Histological preparation techniques, Rose, Staining.

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INTRODUCTION

Rose flower is designated as the queen of flowers. Its pleasant aura has facilitated its use in cosmetics. It is also used in food industries as a flavoring agent. The genus *Rosa* also belongs to the Rosaceae family that comprises 4,828 species of flowers. It is available in a wide range of colors like white, yellow, pink, and red. The flowers hibiscus and rose belong to the kingdom Plantae. A recent study showed that *Hibiscus sabdariffa* has been used for histological staining tissues of lymph node and kidney.^{1,2} Studies using rose extract (anthocyanins) to stain biological tissue were found to be limited.³ The natural extract dyes are eco-friendly and possess the advantage of being nonhazardous.⁴

Use of natural rose extract for staining of histopathologic sections would be economical as they are easily available. Therefore, rose extracts can be used as a substitute for synthetic eosin stain.^{1,2}

In the present study, two methods for rose extraction, namely maceration and soxhlet, were compared with respect to their staining efficacy, the staining of REM and rose extracts without mordant, H&R with H&E on normal and pathological tissues were also compared.

MATERIALS AND METHODS

Material Collection

This study was conducted on formalin-fixed paraffin-embedded blocks (normal and pathological tissues of OSCC) from the archives of the Department of Oral Pathology, Faculty of Dental Sciences, Ramaiah University of Applied Sciences, Bengaluru, Karnataka, India. The study was conducted for a period of 6 months. Ethical clearance was obtained from the college ethical clearance board.

In the present study, rose petals were dried and powdered. The extraction of powdered rose was done by maceration and soxhlet method.^{4,5}

Procedure of Rose Extraction

Maceration Technique

A quantity of 15 g of finely powdered rose petals was dissolved in 500 mL of 95% ethanol. The preparation

was left undisturbed for 48 hours. The filtrate obtained was used to stain the tissues.⁴

Soxhlet Technique

A quantity of 500 gm of finely ground rose was weighed and placed in the soxhlet apparatus, and 500 mL of 95% alcohol was brought to boiling point. The vapor containing the extract was collected. The Soxhlet extraction procedure was completed by 3–4 days.⁵

Tissues Considered in the Study

Forty paraffin-embedded tissue blocks of 20 normal oral mucosa and 20 pathological tissues (OSCC) were taken in the present study.

Preparation of the Staining Solution

The staining solution was prepared as per the previous study conducted by Sridhara et al.⁴ Both rose extract solutions were filtered using a Whatman filter paper. However, pH and concentration were standardized using trial and error method. The pH of the staining solution was adjusted to 6.2. The solution was stored in polyethylene bottles at -4°C . As for the preparation of solution with mordant, 10 g of potassium alum was added before dilution to 100 mL.

Staining Technique

Sections with a thickness of 4 μ from 20 blocks of paraffin-embedded normal tissues and pathologic tissues were taken. The slides were stained using:

- Routine H&E stain
- Hematoxylin followed by counterstaining with rose extract solution without the mordant
- Hematoxylin followed by counterstaining with rose staining solution with the mordant (potassium alum).^{2,5-7}

The tissue sections were deparaffinized, treated with xylene (clearing agent), and 60 and 70% alcohol for 5 minutes each. Table 1 lists the protocol followed for staining.

Table 1: Staining protocol

H&E		H&R	
Reagent	Routine	Reagent	Routine
Water	10 minutes	Water	10 minutes
Hematoxylin	15 minutes	Hematoxylin	15 minutes
Bluing	10 minutes	Bluing	10 minutes
Acid alcohol	1 dip	Acid alcohol	1 dip
Water bath	10 minutes	Water bath	10 minutes
Eosin	1 minute	REM	20 minutes
Absolute alcohol	1 dip	Absolute alcohol	1 dip
Xylene	10 minutes	Xylene	10 minutes

Examination of the Slides

The staining efficacy of REM and rose without mordant, in normal and pathological tissues, was compared by three observers. The criteria followed to determine the staining ability were based on histomorphologic appearance like nuclear staining, cytoplasmic details, staining intensity, and contrast/clarity. The slides were then graded based on the staining intensity of rose extract obtained by different techniques. Each of the criteria was given a score of 0 and 1 based on the ability to appreciate the stained section. The sum obtained on scoring individual slides determined if the stained slides were 0 = poor, 1 = satisfactory, 2 = good, and 3 = excellent (Table 2). The criteria given by Buesa et al, 2000 for grading stained tissues was modified in the present study.^{4,8,9}

Statistical Analysis

Chi-square test was employed to determine the statistical significance.

RESULTS

Comparison of the staining intensity of the extracts prepared by the maceration and the soxhlet method of extraction showed comparable staining efficacy (p value=0.78) (Figs 1A, B and Graph 1).

Comparison of REM and rose extract without the mordant showed that the staining with REM was superior to staining with rose extract without a mordant. The statistical test showed a statistically significant difference (p value = 0.04) (Figs 1C, D and Graph 1).

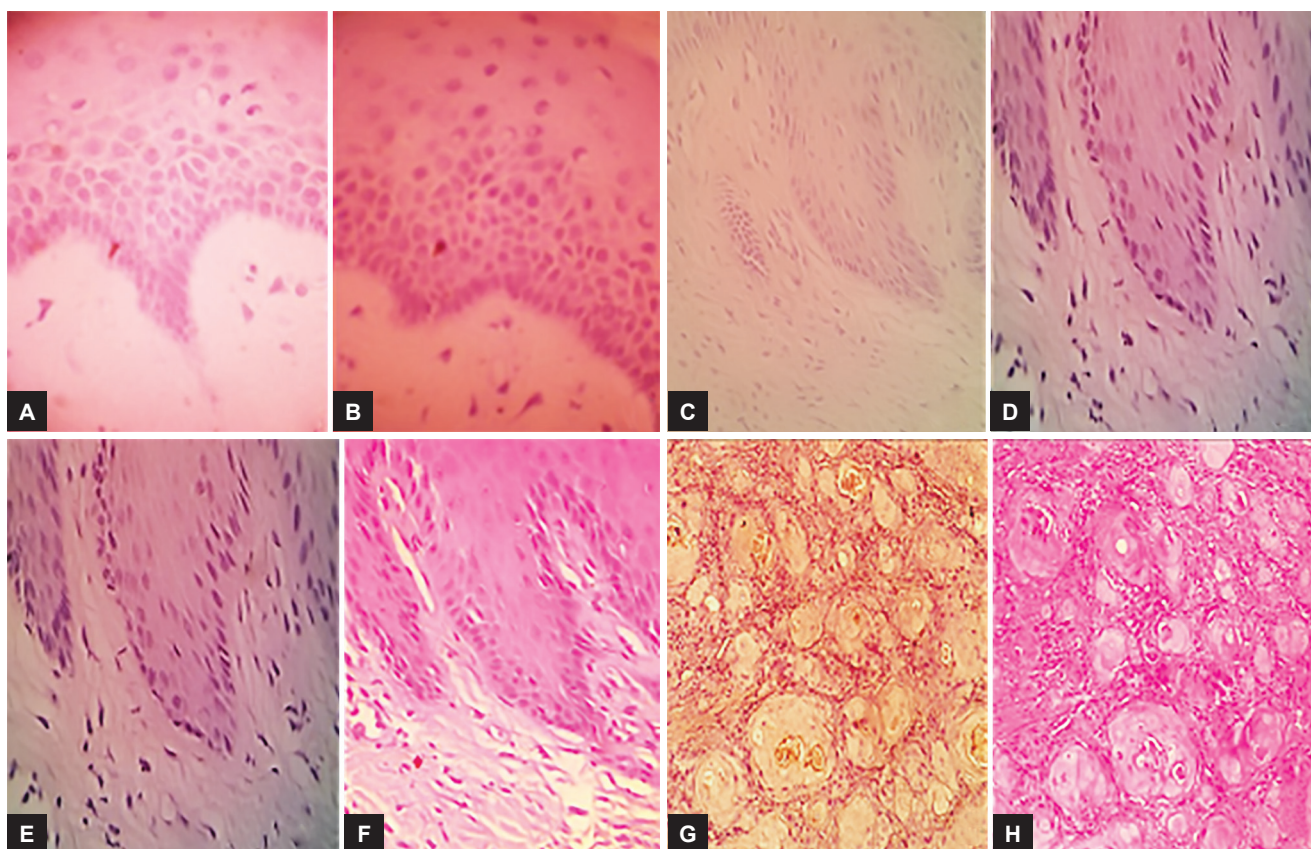
Comparison of the staining efficacy of rose extract with that of eosin in normal as well as in the OSCC tissues showed comparable results (p value = 0.51 and 0.91, respectively) (Figs 1E to H and Graph 1).

DISCUSSION

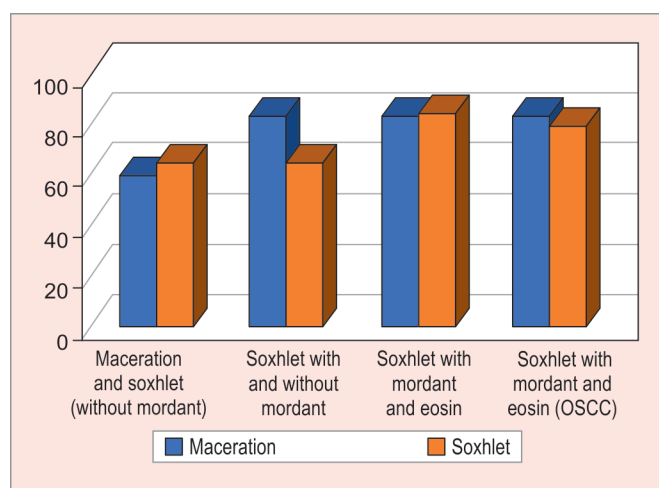
Staining of tissues is essential for adequate histopathological diagnosis. Use of synthetic stains can cause health

Table 2: Scoring criteria for the assessment of stained sections

Overall histologic appearance	0 or 1
Staining intensity	0 or 1
Contrast	0 or 1
Nuclear staining	0 or 1
Cytoplasmic staining	0 or 1
Final score	
Poor	0
Good	1
Satisfactory	2
Excellent	3



Figs 1A to H: (A) Comparison of the staining efficacy of rose extract prepared by maceration method; (B) Rose extract prepared by Soxhlet method; (C) Rose extract without the mordant; (D) Rose extract with the mordant; (E) Rose extract with eosin stain in normal tissue; (F) Hematoxylin and eosin stain in normal tissue; (G) Rose extract with eosin stain in OSCC tissues; (H) Hematoxylin and eosin stain in OSCC tissues



Graph 1: Comparison of the three objectives (normal and OSCC tissues)

issues and produce environmental hazards. Exploring the staining potential of naturally available stains could provide a substitute for synthetic stains like eosin. In this study, we have used rose extracts to stain tissues. Similar to other studies, the rose petals were dried and coarsely powdered.^{4,10,11} In the present study, coarsely powdered rose petals were used for extraction unlike other studies^{6,12,13} as finely powdered preparation slows down the process of filtration.

Principles of staining suggest that tissue components and the dye interact through ionic bond and establish an electrostatic attraction between dissimilar ions. In majority of histological staining procedures, an ionic bond is appreciated between the stain and the tissue section. The process of the binding of tissue with the stain depends on the bond between the dye and the tissue components. There are various factors affecting the staining of tissue which includes concentration of dye, time of action on the solvent, its aqueous or alcoholic nature, and its pH.^{14,15} Rose extracts have been used as a counterstain to hematoxylin, as it contains anthocyanin pigments that impart pink to red color to the tissues.^{3,16} It has been tried earlier by Kumar et al.³ to stain *Platyhelminthes* tissues, such as *Fasciola gigantica*, *Gastrothylax crumenifer*, *Taenia solium*, and *Moniezia expansa*. Rose extracts were also used to stain angiospermic tissue, animal tissue, paramecia, and cottony white fungus in studies conducted by Korade et al.⁵ As rose extracts were never tried earlier to study oral tissues, in this present study, rose extracts were taken to compare with eosin in normal and pathological oral tissues.

Traditionally, to obtain extracts from natural sources, maceration or soxhlet methods are employed.^{4,5} Earlier studies conducted by Korade et al.⁵ on aqueous extracts of henna, hibiscus, madder, fire flame bush, rose, and

bougainvillea used only soxhlet method of extraction to stain angiospermic and animal tissues. Kumar et al³ in their study used 96% ethanoic extracts prepared by the maceration method to stain the *Platyhelminthes* specimen. To our knowledge, comparison between the two methods of extraction (maceration and soxhlet) in terms of staining efficacy was not attempted earlier. In the present study, tissues stained by soxhlet method of extraction fetched better results than the maceration method. In Soxhlet technique, greater yield of anthocyanin pigments was obtained due to heat application that enhanced the staining efficacy in contrast to maceration technique.

Based on the trial and error procedure, pH and the time required to stain the tissues with the rose extracts was standardized. In the present study, pH of 6.2 fetched better staining efficacy. Addition of mordant (i.e., potassium alum in the ratio of 1:10) to the rose extract showed better results than that of rose extract without the mordant. It was also found that there is comparable staining efficacy with that of eosin stained tissues.

Earlier studies have shown that acidity, alkalinity, and mordant affect some stains. In a study by Elbadawi in 1976, the need for mordants in certain histochemical reactions has been stressed.¹⁷ Mordant is a substance that binds the stain on tissue sections by forming a coordination complex, thereby enhancing the retention property of the stain to the tissue.¹⁸ He also reported that ferric chloride as mordant was necessary in Verhoeff's iron hematoxylin stain. In another study conducted by Alawa et al.,¹⁹ potassium alum was used as a mordant to improve the staining affinity of henna to the rat brain tissue. Similarly, in the present study, we incorporated potassium alum as a mordant in the ratio of 1:10 and found better staining efficacy than staining of tissue with rose extract alone.

In the present study, REM has shown better staining efficacy in normal oral tissues, which is similar to studies conducted by Korade et al.⁵ on animal tissues. The staining efficacy was evaluated based on histomorphologic appearance like nuclear staining, cytoplasmic details, staining intensity, and contrast/clarity, which showed comparable staining efficacy with that of eosin. On pathological tissues like OSCC, features like nuclear hyperchromatism, enlarged nucleoli, cellular pleomorphism, increased nuclear cytoplasmic ratio, irregular epithelium stratification, breach in basement membrane, and the presence of keratin pearls can be similarly appreciated in both H&E and H&R stains. To our knowledge, this was the first study on staining pathological oral tissues with rose extracts.

Although rose extract is a natural stain, it tends to achromatize over a period of time. The shelf life of the slides with mordant was comparable to that of eosin, when reviewed after 6 months. Though the attempt of

this study was to use natural stains, we could not find a natural mordant, hence used potassium alum. Further research to discover a natural mordant and testing its efficacies on various pathologies may be beneficial.

CONCLUSION

Tissue sections stained by the soxhlet method fetched better and statistically significant results than the maceration method. It was also noticed that tissue components stained with mordant provided greater staining efficacy than rose extracts alone. Normal and pathologic tissues stained with REM fetched comparable results with eosin.

Recently, advanced techniques for extraction like microwave-assisted extraction, ultrasound-assisted extraction, and supercritical fluid extraction have been introduced. Use of these advanced extraction techniques, addition of appropriate natural mordant for better staining, and greater shelf life would be beneficial so that it could be used as a counterstain to hematoxylin in future.

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