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ORIGINAL RESEARCH

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Lawsonia inermis (henna) extract: A possible natural substitute to eosin stain

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ABSTRACT

Background: The extensive evolution of histopathology can be mainly attributed to the availability of a wide array of stains. Staining has made possible the identification of various tissue structures under microscope aiding in appropriate diagnosis. However, the present era of increasing importance to ecology has necessitated the requisite for natural dyes. Unlike synthetic dyes, natural dyes are less toxic, biodegradable, and are eco-friendly. Hematoxylin and eosin are the routinely used stains in histopathology wherein eosin is a synthetic stain. Various attempts have been made to substitute eosin with a natural dye, one among which is *Lawsonia inermis* (Henna). Nevertheless, the staining efficacy of henna extract on formalin-fixed paraffin-embedded oral tissues, as a counterstain to hematoxylins is yet to be determined.

Aim: The study aims to analyze the use of *Lawsonia inermis* extract as a possible substitute for eosin stain in paraffin-embedded oral tissues.

Methodology: The coloring component of dried leaves of henna was extracted using maceration and Soxhlet method. Four micro sections of 20 paraffin-embedded oral tissues of normal oral mucosal tissues and Oral squamous cell carcinoma (OSCC) tissues each were stained using the extract and counterstained to hematoxylin and studied for its staining efficacy.

Statistical Analysis: Chi-square test was done and noted for any significant results.

Results: On comparing the staining efficacy of henna while using different extraction methods, the Soxhlet method was better than the maceration method by 20%, however, statistically the results were insignificant. Staining efficacy of henna with and without the mordant was statistically significant wherein staining with mordant gave inferior quality stain. When henna stain was compared to eosin, comparable results were found, with eosin being slightly better by 15%.

Conclusion: In this era of increasing importance to ecology, henna may well prove to be an effective alternative to eosin in histological sections of normal and pathological tissues as both the stains gave comparable results.

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Introduction

Lawsonia inermis Linn, commonly known as Henna, is a shrub belonging to the Lythraceae family. It is seen as a perennial shrub in sub-tropical and tropical areas of Middle East, Africa, Southern Asia, Northern Australia, and other semi-arid areas. It is widely used to augment beauty and as a commercial crop [1,2]. One of its large-scale use includes those for cosmetic purposes as pigments to color hair and nails imparting a reddish yellow tint [1,2]. Other uses are namely, in textile industries for dyeing wool and nylon, traditionally practiced medicinal plant for the treatment of various ailments. Of late, attempts have been made to use henna as a biologic stain for plants and micro-organisms [3].

The staining property of henna is mainly attributed to a naphthoquinone compound named as lawsone which is seen in abundance in the dried leaves. The compounds in these dried leaves impart a brown color as they have chemical properties that are analogous to tannic acid and thus its name hennotannic acid [4]. Despite henna's wide use in

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cosmetics and textile industries, limited attempts have been made to note its use as a histologic stain on fixed paraffin-embedded human tissues.

Stains are basically dyes which have been modified for its use in biology. These colored compounds yield color to a tissue based on its affinity to the substrate present in them. Mordants may be required for few stains in order to improve the fastness of the stain to the specific tissue. Up until the mid of the 19th century, nature had been the sole source of dyes. Most of them were from vegetable extracts with only some from animal sources. [3] The first man-made synthetic dye was discovered by an English chemist named William Henry Perkin in 1856. Since then thousands of synthetic dyes have come up replacing natural dyes as they offered a wide range of colors and proved to have better properties compared to dyed materials [3].

There is an increasing importance to ecology in the present era which has necessitated the requisite for natural dyes. Unlike synthetic dyes, natural dyes are less toxic, biodegradable, and are eco-friendly [1,4,5]. Haematoxylin and Eosin (H&E) are the routinely used stains in histopathology wherein eosin is a synthetic stain. Since eosin is a synthetic stain, attempts have been made to replace them with natural dyes. Various natural dyes such as hibiscus, turmeric, Rosa indica, etc. have been tried for their use as biological stain other than henna [6–8].

Studies on animal tissues have suggested henna to be an acidic stain with increased affinity for sclerotic proteins such as collagen, keratin, and elastin which constitutes connective tissue and muscle fibers. It has been proposed that the staining mechanism of *Lawsonia inermis* is hinged on ionic interactions occurring between the phenolic group in dye and the amino end-group in tissue proteins that forms a major component of the connective tissue. Such ionic interactions are also accompanied by no ionic interactions such as polymerization and thus giving metachromatic properties to the dye [4]. However, the use of henna as a counterstain to hematoxylin in formalin-fixed paraffin-embedded (FFPE) oral tissues is yet to be explored. In the light of the known properties of henna stain from existing literature, the present study aimed at exploring the possible use of henna stain as a substitute to eosin.

Aim

The study aims to analyze the use of *Lawsonia inermis* extract as a possible substitute for eosin stain in paraffin-embedded oral tissues.

Objectives

1. To assess the staining efficacy of henna extract by employing two different methods (Maceration and Soxhlet method).
2. To determine the staining efficiency of henna extract with and without the use of mordant.
3. To evaluate the staining efficacy of henna extract in comparison with eosin stain in normal oral mucosa and oral squamous cell carcinoma FFPE tissues.

Materials and methods

Plant collection and extraction

Fresh leaves of *L. inermis* (henna) were collected from Sadvaidyashala garden, which are dried and powdered into coarse granules and taken for extraction.

Extraction was done using:

1. Maceration method
2. Soxhlet method

Maceration method

One hundred gram of dried powdered leaves was soaked in 1,000 ml of water and left for 24 hours. The solution was then filtered using Whatman filter paper. This filtrate was then concentrated using an evaporator and a yield of 18.6% was obtained.

Soxhlet method

One hundred gram of the dried powdered leaves was extracted in a Soxhlet apparatus with distilled water for about 72 hours, or until the solvent ran clear. Extracts were then concentrated in an evaporator with a yield of 17.6%.

Preparation of the staining solution

The staining solution was prepared as per a previous study conducted by Alawa et al. [4], however, pH and concentration were standardized using the trial and error method. To prepare the staining solution, 2 g of henna dye extract, 5 ml of ethanol were dissolved in 50 ml of distilled water. This solution was then shaken vigorously for 1 minute and allowed to stand for 30 minutes in order to permit proper dissolution of the henna dye. After which, 4 ml of glacial acetic acid was added and the solution was diluted to 100 ml with distilled water and mixed. The solution was then filtered using a Whatman filter paper. The pH of the staining

Table 1. Staining protocol.

Hematoxylin and eosin		Hematoxylin and henna	
Reagent	**Routine**	**Reagent**	**Routine**
Water	10 minutes	Water	10 minutes
Hematoxylin	10 minutes	Hematoxylin	20 minutes
Blueing	10 minutes	Blueing	10 minutes
Acid alcohol	1 dip	Acid alcohol	1 dip
Water bath	10 minutes	Water bath	10 minutes
Eosin	1 minutes	Henna extract	1 hour
Alcohol	1 dip	Alcohol	1 dip
Xylene	10 minutes	Xylene	10 minutes

solution was adjusted to 7.5. A few crystals of thymol were also added to prevent fungal growth. The solution was stored in polyethylene bottles. As for the preparation of the solution with mordant, 10 g of potassium alum was added before dilution to 100 ml.

Tissue selection and staining

Histopathologically confirmed FFPE blocks of normal oral mucosa (20) and oral squamous cell carcinoma (20) were selected. Inclusion criteria included those tissues with adequate epithelium and connective tissue. Four micro sections of each block were taken based on the requirement for each objective. Tissues sections that had artifacts were excluded. Ethical clearance was obtained from the ethical clearance committee. The tissue sections were subjected to deparaffinization followed by treatment with 70% and 60% alcohol for 5 minutes each. Table 1 enlists the protocol followed for staining.

The slides were stained using the following stains:

- Routine H&E staining
- Hematoxylin followed by counterstaining with henna staining solution without the mordant (potassium alum)
- Hematoxylin followed by counterstaining with henna staining solution with the mordant (potassium alum)

Table 2. Scoring criteria for assessment of stained sections.

Scoring criteria for assessment of stained sections	
Parameters	Score
Overall histologic appearance	0 or 1
Nuclear staining	0 or 1
Cytoplasmic details	0 or 1
Staining intensity	0 or 1
Contrast	0 or 1
Final score	
Poor	0–2
Good	3
Satisfactory	4
Excellent	5

Assessment of stained sections and statistical analysis

The stained slides were blinded and observed by three observers. Table 2 lists the scoring criteria for the assessment of the stained sections as modified by Buesa et al. [9]. The stained sections were given a score from 0 to 5 wherein parameters such as overall histologic appearance, nuclear staining, cytoplasmic details, staining intensity, and contrast were considered. Chi-square test was done and noted for any significant results.

Results

Staining efficacy of henna when extracted using two different methods of extraction

Based on previous trials, pH, concentration, and the time required for adequate staining with henna were standardized (data not shown). We wanted to check if different methods of extraction had an effect on the staining efficacy of henna when used as a counterstain to hematoxylin. On evaluation of the stained slides, henna stained the keratin, cytoplasm of cells, and the collagen fibers as a light brown color (Fig. 1). The extract obtained from the Soxhlet method showed better staining quality in comparison to maceration method. Since results showed that henna extracted with Soxhlet methods

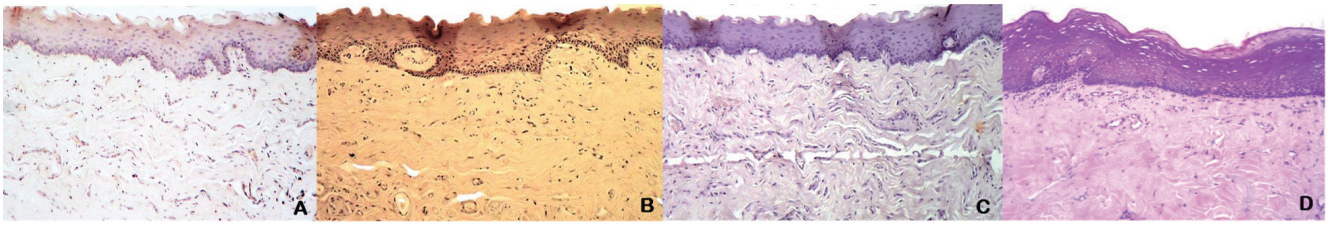

Figure 1. (A) H&H (Soxhlet) stained normal oral mucosa (40×). (B) H&H (maceration) stained normal oral mucosa (40×). (C) H&H (with mordant) stained normal oral mucosa (40×). (D) H&E stained normal oral mucosa (40×).

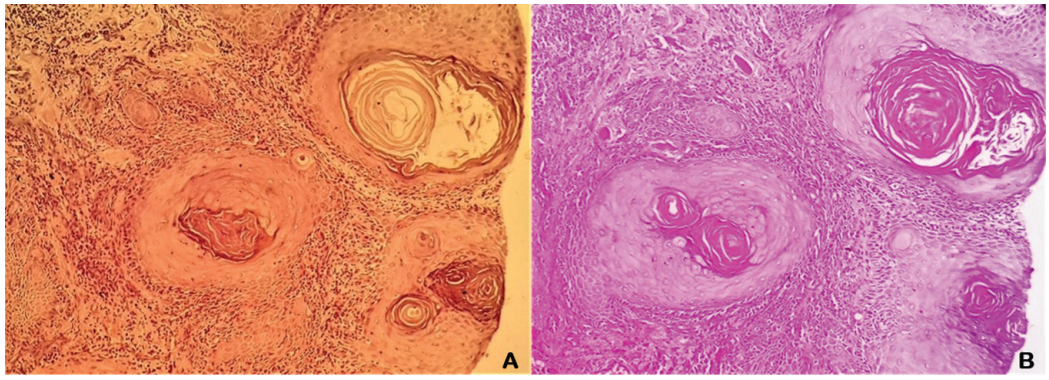

Figure 2. (A) H&H (Soxhlet) stained oral squamous cell carcinoma (10×). (B) H&E stained oral squamous cell carcinoma (10×).

elicited better results, we proceeded to use extract obtained from the above-mentioned method for the rest of the study.

Staining efficiency of Henna with and without mordant

We wanted to explore if henna staining improved when used with mordant such as potassium alum, which had been previously used by Alawa et al. [4]. However, we found that the staining quality of henna was greatly reduced when used with mordant (Fig. 1). As henna with mordant showed poor results, we continued to use henna without mordant for further investigations.

Comparison of staining efficiency between Henna and Eosin when used as a counterstain to Hematoxylin

On comparison between Hematoxylin and Henna (H&H) and H&E, the similarity was observed in staining of the various tissue elements of both normal oral mucosal tissues (Fig. 1) and oral cancer tissues (Fig. 2). Henna stained tissues by imparting a brown color to the cytoplasm of cells, keratin, collagen fibers as well as red blood cells. Evaluation of H&H showed clear morphologic identification of epithelium as well as the connective tissue elements. There existed a good contrast between henna and hematoxylin as henna stain did not mask the hematoxylin color. However, the time duration required for the H&H staining is much longer than that of H&E. H&E showed slight superiority in staining due to its better staining intensity when compared to H&H. The summary of overall scores, comparison between groups and respective *p* values have been presented in Figure 4.

Discussion

Staining has become a vital part in histology. However, it involves the use of synthetic stains which pose a possible threat to the ecosystem. Hence, we thought of exploring naturally available stains that could substitute eosin. Henna has been widely used as an ornamental stain for hair, skin, and nails giving a reddish orange to brown color. This staining ability of henna can be attributed to its chemical composition. Henna contains a wide group of phenolic compounds, one among which is a dye that falls under the naphthoquinone group of compounds named "Lawsone." Moreover, Naphthoquinones are one among the many compounds known for its high reactivity towards amino acids. There have been reports demonstrating naphthoquinones yielding a brown to purple pigment on its reaction with cysteine and proteins. It is also a known fact that proteins are one of the main constituents of any biological tissue. This explains the reason for tissue components to yield a brown color in the present study. This is in favor with other studies that incorporated henna extract to stain rat brain tissues [4], angiospermic stem tissues [1], onion epidermal cells [10], and different bacteria [2,3].

Any natural herb needs to be subjected to the process of extraction to obtain the extract. The best suitable extraction method for a natural herb will depend on their phytochemical properties. Different authors have employed various methods for the extraction of henna with maceration and Soxhlet being two of the commonly employed techniques. However, in our study, we wanted to investigate if extraction methods could affect the staining efficacy of the henna extract. We employed maceration and Soxhlet methods as they were commonly preferred. Dried leaves of henna were preferred over fresh leaves based on reports that suggested the increased content of

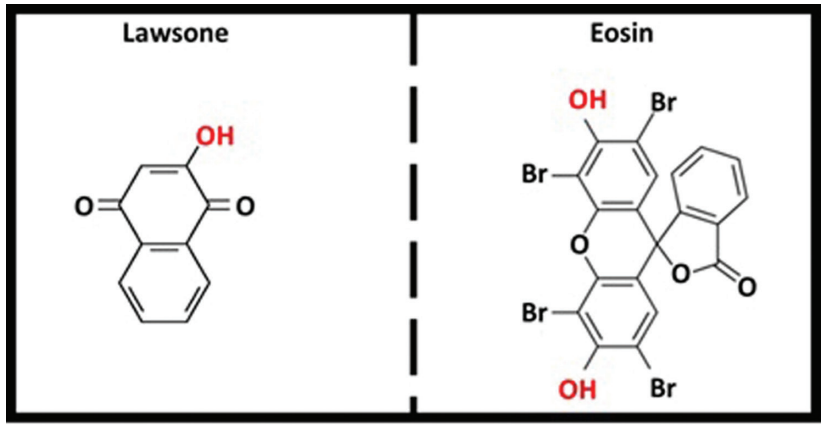

Figure 3. Similarity between the molecular structure of lawsone and eosin with both of them containing hydroxyl groups.

lawsone in dried leaves [11]. We found a statistically significant difference in the staining efficacy of extracts obtained from maceration and Soxhlet method wherein superior staining was observed with the extract obtained from the later. The better staining efficacy could be attributed to the greater amounts of lawsone present in the extract.

Soxhlet extraction is the classical technique employed in solvent extractions to obtain various compounds from plants. Studies have proved its efficiency in producing greater amounts of the compound with a lesser quantity of solvent [12–14]. A study on the extraction of lawsone using various methods suggested that large amounts of lawsone were obtained via Soxhlet extraction when compared to other methods [15]. The present study is the first of its kind to explore the effect of extraction methods on the staining efficacy of henna on tissues. However, further investigations employing other methods such as accelerated solvent extraction and ultrasonic assisted extractions are required in this field.

Based on previous trials, pH and time required for an acceptable stain were determined and better staining intensity was obtained at a pH of 7.5 and time duration of 45 minutes. Lawsone, also called as hennotannic acid is an acidic stain that requires neutral or slightly alkaline pH in order to facilitate the dissociation of the negative ionic groups. The negative ionic groups bind to the acidophilic structures of the tissues such as the cytoplasm, extracellular matrix, collagen fibers, and muscle fibers [16]. However, increase in pH above 8 reduces the staining intensity as lawsone is unstable under alkaline condition [17].

Mordants have been employed to improve the staining intensity of few stains. Studies on rat brain tissues have reported the use of potassium alum as a mordant for henna which improved the affinity of the dye to the tissue [4]. However, in the present study, the addition of potassium alum resulted in a pronounced drop in pH. A similar observation was reported in a study conducted by Chukwu et al. [3]. Decrease in pH directly correlated with the decrease in the dissociation of negative ion groups which in turn reduces the binding to the acidophilic tissue structures [16]. This was obvious in the present study as the sections stained using henna with mordant and hematoxylin appeared to have taken up only hematoxylin with no evident staining by henna.

Eosin has been widely used as a counterstain to hematoxylin. Any counterstain's prime objective is to provide a good contrast to the principle stain without hampering the staining of the same. This allows a better distinction between the nucleus and other components such as cytoplasm. One of the reasons for exclusively choosing henna among the other natural stains in our study is its closeness to eosin in terms of physicochemical properties. Like eosin, henna also consists of a hydroxyl group (Fig. 3). This acidic part of the stain shows an affinity towards the amino end-group found in tissue proteins that form a major part of the connective tissue. Studies on animal tissues have suggested henna to be an acidic stain with an increased affinity for sclerotic proteins such as collagen, keratin, and elastin which constitutes connective tissue and muscle fibers. Such ionic interactions accompanied by non-ionic interactions (like polymerization) give metachromatic properties to the dye [4]. These properties of henna are responsible for its staining in tissue structures like keratin, cytoplasm, collagen fibers, muscle fibers, and the like. This also explains the increased staining affinity of henna towards keratin pearls in Oral squamous cell carcinoma (OSCC) tissue sections.

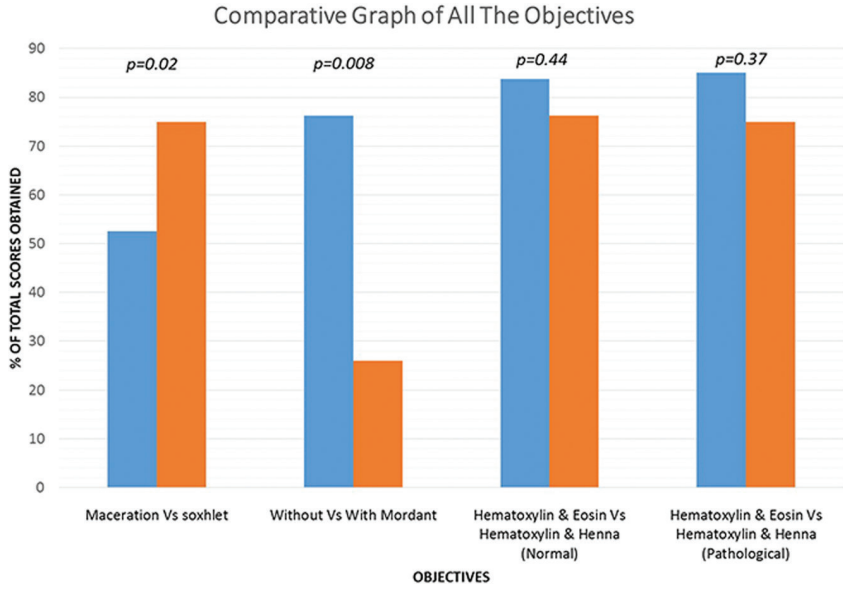

Figure 4. Graph comparing the percentage of the total score obtained in each group with regard to all the objectives ($p < 0.05$ was considered to be statistically).

Conclusion

With the rising importance being given to the ecosystem, there exists a need to replace synthetic dyes with naturally available dyes due to their eco-friendly nature. With this study, it can be inferred that henna could be used as a possible substitute to the widely used eosin stain. However, further investigations are required to discover suitable mordant and/or accentuators which would increase the staining intensity as well as reduce the staining time, thus making henna a potent stain.

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