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A histopathological correlative assessment of cytoplasmic staining by aqueous extracts of hibiscus and turmeric with eosin on 70 tissue samples

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***Abstract*---**Dyes are the substances which are used for imparting color. They are mainly used in food, textiles, paint, leather & printing industries. Dyes are extensively used in histopathology and cytological examination in oral pathology. Hematoxylin and Eosin stains are primarily used for differentiating between cell nucleus and cytoplasm. However, Eosin been a xanthene derivative is synthetic in nature and hence is known to be carcinogenic. Replacement of chemical dyes with natural ones is the need of the hour in this era of global warming and environmental pollution. The aim of the study was to determine the efficacy of aqueous extract of turmeric and hibiscus as a cytoplasmic

stain replacing eosin and to formulate a natural staining agent using Turmeric and Hibiscus. Seventy paraffin-embedded tissue blocks were selected.Three sections were made from each block. One section was stained with conventional H&E method (used as control), second section was stained with 10 % aqueous turmeric extract while third section was stained with 75% aqueous hibiscus extract.Tissue were evaluated using following parameters:- Nuclear staining, Cytoplasmic staining, Cell morphology, Clarity of staining, Uniformity of staining and Background staining. The results were recorded by 2 independent oral pathologists. Kappa test and Chi-square test were used for comparison of qualitative data. Inadequate Nuclear staining was found in 1.43% and 7.14% of the slides in Eosin Turmeric and Eosin Hibiscus group ($p>0.05$). Inadequate cytoplasmic staining was found in 8.57% and 5.71% of the slides in Eosin Turmeric and Eosin Hibiscus group ($p>0.05$). Inadequate and patchy staining was found in 2.86% and 8.57% of the slides in Eosin Turmeric and Eosin Hibiscus group ($p>0.05$). Inadequate clarity of staining was found in 11.43% and 21.43% of the slides in Eosin Turmeric and Eosin Hibiscus group ($p>0.05$). Background staining was reported among 15.71% and 17.14% of the slides in Eosin Turmeric and Eosin Hibiscus group ($p>0.05$). Cell morphology was present among 5.71% and 17.14% of the slides in Eosin Turmeric and Eosin Hibiscus group ($p<0.05$). Aqueous stain of turmeric and hibiscus obtained from the rhizomes of Curcuma Longa and the petals of Hibiscus Rosa Sinesis respectively can be used as an alternate to eosin as an cytoplasmic stain.

***Keywords*---**hematoxylin, eosin, turmeric, hibiscus, background staining.

Introduction

Dyes are the substances which are used for imparting color. They are mainly used in food, textiles, paint, leather & printing industries. Dyes can be natural or synthetic on the basis of its origin. Making dyes from herbals is not anew thing to the mankind. Many flowers, roots, leaves or bark of some herbal species have been used mostly by boiling, powdering and mixing with other materials to form natural dyes. Whereas synthetic dyes are commercial dyes are simpler and ready to use. In oral pathology, dyes are primarily used in Histopathology and cytological examination. Dyes which are mainly used are Hematoxylin and Eosin stains. Hematoxylin stains the acidic components of the cell (nucleus), giving them dark violet or blue color and Eosin stains the basic components of the cell (cytoplasm), giving them pink color. Eosin been a xanthene derivative is synthetic in nature and hence is known to be carcinogenic. Moreover its proper disposal and ever increasing cost pose a threat to many laboratories. Hence, we need to develop safe, non-toxic and organic stains to promote an eco-friendly working environment. Replacement of chemical dyes with natural ones is the need of the hour in this era of global warming and environmental pollution. Carmine, turmeric, henna, wing fruit, china rose are few natural dyes which gives a

contrast color with hematoxylin and can be used easily as a replacement for eosin.

Since, the 17th century, Leeuwenhoek used madder, indigo and saffron to stain tissues and used rudimentary microscopes to study them. Many natural dyes have been used in the past such as Carmine:Scarlet dye obtained from the ground bodies of cochineal beetles, used in the cytology as early as 1770 by John Hill. [1,2] Henna (Lawsoniainermis) gives a red orange dye molecule, "lawsone", a hennotanic acid. This hennatannic acid has affinity towards proteins and also has a fast-dyeing property. Recent research has proven its efficacy as a counter stain in Gram staining. In Gram staining it has been used in an extract oxidized with potassium permanganate.[3] Wing fruit (Pterocarpusosun): is a forest tree ,belonging to papillionaceae family which gives off a red pigment. This red pigment has proven promising results in staining collagen fibres, red blood cells and muscles.[4] Beet root (Beta vulgaris) extract has shown promising results with vinegar as a mordant as a potential in replacing eosin.[5] Red rose (Rosaesinenesis) contains anthocyanin pigment which impart a pink to red colour to the tissues. Rose extracts are also used to stain angiospermic tissue, tissue, paramecia and cottony white fungus. [6] Turmeric belongs to Zingiberacae family and has curcumin, which are natural phenols responsible for turmeric's yellow colour.This curcumin is responsible for staining. Curcumin was first isolated by Vogel in 1842, comprising of curcurmin1 (94%), curcumin2 (6%) and curcumin 3 (0.3%). Its chemical structure was determined by Roughley and Whiting in 1973.[7,8,9] Hibiscus sadbariffa, also called Roselle or kajarat, belongs to the family Malvaceae. Its major pigment is hibiscin, recognized as daphniphylline. The commercially important part of the plant is the calyx (sepals). It is red in color and acidic in pH. The extract can be used as a dye for biological staining.[10].Hence, in our present study we used aqueous extracts of turmeric and hibiscus because of its easy availability, economic viability and ease of stain preparation.

Materials and Methods

70 paraffin-embedded tissue blocks were retrieved from archive of the Department of Oral & Maxillofacial Pathology and Oral Microbiology of Surendera Dental College and Research Institute, Sriganganagar, Rajasthan. 70 samples included 10 gingival enlargements, 10 odontogenic cysts, 10 benign epithelial lesions, 10 oral squamous cell carcinomas, 10 salivary gland tumours, 10 benign connective tissue tumours, and 10 mucocutaneous lesions. Eosin powder-Spirit soluble, Turmeric aqueous extract and Hibiscus aqueous extract solution were made (Extract solution formulation procedure Table 1). Three serial sections were made from each tissue sample. One section was stained as per regular protocol for hematoxylin and eosin staining (in Harris hematoxylin for 6 minutes and eosin in 15minutes) to be used as control. The second section was stained by 10 % aqueous extract of turmeric for 120 minutes instead of eosin. The third section was stained by 75% aqueous extract of hibiscus instead of eosin for 120 minutes (Staining protocol Table 2). 210 sections were blindly examined and decoded by two histopathologists. Following criterias were used:- Nuclear staining, Cytoplasmic staining, Cell morphology, Clarity of staining, Uniformity of staining and Background staining (Adequate =1 and inadequate =0)[13] (Table 3). Kappa test

and Chi-square test were applied using SPSS software version 22.0. The data was analyzed and the test results tabulated and evaluated.

Results

Hematoxylin and Eosin staining were carried out on 70 tissue sections to be used as control, 70 tissue sections were stained with turmeric extract and 70 tissue sections were stained with hibiscus extract. The tissue sections were evaluated by two independent oral pathologists. Nuclear staining, cytoplasmic staining, clarity of staining, uniformity of staining, background staining and cell morphology were the criteria's used to assess the staining quality of the tissue sections. Data was analyzed by SPSS 22.0 software (SPSS Inc., Chicago, IL, USA) using kappa and chi square test. In our study, two investigators reported and recorded their analysis of slide. We checked the reliability between the two investigators using kappa test and it was found to be 0.86 which was excellent. The comparison of nuclear staining among the study groups revealed inadequate staining in 1.43% and 7.14% of the slides in Eosin Turmeric and Eosin Hibiscus group respectively,and chi square test revealed p value>0.05,hence it proved statistically insignificant. When nuclear staining was compared among Eosin Turmeric and Eosin Hibiscus group using chi square test, inadequate staining was revealed in 8.57% and 5.71% of the slides in Eosin Turmeric and Eosin Hibiscus group respectively. When cytoplasmic staining was compared among Eosin Turmeric and Eosin Hibiscus group using chi square test, it was found to be statistically insignificant as $p>0.05$.

Comparison of staining uniformity among the study groups revealed inadequate and patchy staining in 2.86%, 97.14% and 8.57%, 91.43% of the slides in Eosin Turmeric and Eosin Hibiscus group respectively and staining uniformity was compared among Eosin Turmeric and Eosin Hibiscus group using chi square test, it was found to be statistically insignificant as $p>0.05$. The comparison of clarity of staining among the study groups revealed inadequate clarity of staining in 11.43% and 21.43% of the slides in Eosin Turmeric and Eosin Hibiscus group respectively. When clarity of staining was compared among Eosin Turmeric and Eosin Hibiscus group using chi square test, it was found to be statistically insignificant as $p>0.05$. The comparison of background staining among the study groups. Background staining was reported among 15.71% and 17.14% of the slides in Eosin Turmeric and Eosin Hibiscus group respectively. When presence and absence of background staining was compared among Eosin Turmeric and Eosin Hibiscus group using chi square test, it was found to be statistically insignificant as $p>0.05$. The comparison of cell morphology among the study groups. Cell morphology was present among 5.71% and 17.14% of the slides in Eosin Turmeric and Eosin Hibiscus group respectively. When presence and absence of cell morphology was compared among Eosin Turmeric and Eosin Hibiscus group using chi square test, it was found to be statistically significant as $p<0.05$. The results of Nuclear staining, Cytoplasmic staining, Cell morphology, Clarity of staining, Uniformity of staining and Background staining criteria are shown in Table 3. The results showed no significant difference in Eosin Turmeric and Eosin Hibiscus group ($p>0.05$).However, Eosin proved to be better staining agent in comparison to turmeric and hibiscus in terms of cell morphology ($p<0.05$).

Discussion

Eosin is the name of several fluorescent acidic compounds which bind to with basic, or eosinophilic, components like proteins containing amino acid residues such as arginine and lysine, and form salts which stains them dark red or pink as a result of the action of bromine on fluoresce in. It stains the protein in cytoplasm and also can be used to stain collagen and muscle fibers for examination under the microscopy. It is classified according to IARC (International Agency on Cancer Research) as group 3 (potentially carcinogenic). Eosin is known to be a skin, eye and mucous membrane irritant. Acute exposure to skin may cause irritation, cheilitis and stomatitis. Prolonged skin contact may cause dermatitis. Acute exposure to eyes causes redness, localised pain and irritation.Considering the above ill-effects of Eosin, we intended to formulate a natural organic stain from Hibiscus and Turmeric as a potential replacement of eosin in H&E staining procedure.O G Avwioro et al confirmed the presence of flavonoids, anthraquinone, deoxysugars as the main chemical structure of turmeric aqueous histological dye. They arrived at a conclusion that 1% of aqueous solution of turmeric stained the collagen fibres, red blood cells, cytoplasm with intense deep yellow colour within five minutes.[1]The ability of a dye to stain specific tissue structures is determined by certain factors one of which is acidity of the stain. Acidic structures would be stained by basic dyes whereas basic structures would be stained by acidic dyes.[11]

Based on the above fact, we came to a conclusion that turmeric extract is an acidic dye as it stain collagen fibres, red blood cells and cytoplasm, proving the affinity of turmeric extract towards the cytoplasm. Similar comparable results were seen in our study, although there was no addition of any mordant nor acids in the turmeric extract, promising results were seen with the application of 10% aqueous extract, which stained the collagen fibres, red blood cells, and the cytoplasm intense yellow color. In our study, staining was done with 10% aqueous extract of turmeric and promising results were seen. Skeletal muscles were adequately stained yellow, revealing a detailed description of the collagen fibres and the striations of the muscles. The results were similar to the study done by Kumar et al, where turmeric had similar staining to eosin for collagen and skeletal muscle fibres.[12] Hema Suryawanshi et al studied the effect of turmeric extract as a stain on eight different tissues such as epithelium, keratin, collagen fibres, muscles, adipocytes, blood vesssels and red blood cells, cartilage and bone.[13] Results obtained were impressive wherein the turmeric extract stained the different tissues in different shades of yellow.The study by Marin Abraham et al proved the efficacy of turmeric extract staining in pathological as well as normal tissues.[14-18] Similar results where noted in our present study where we have used 10% aqueous turmeric extract without mordant, which satisfactorily stained both normal and pathological tissues.[17-20] The morphological and architectural changes of the cells were satisfactorily noted in our study, thus proving the efficacy of turmeric extract as a bio-sustainable alternative to eosin.

Our study used sodium chloride as oxidizing agent instead of ferric chloride and was acidified using 3% glacial acetic acid. We did not use any mordant, possibly paving the way for a new category of aqueous extract of hibiscus–a dye which is

oxidized but not mordanted. Considering the available literature, it is well understood that hibiscus stain is red in colour without oxidation and mordanting, staining acidophilic structures, whereas once oxidized and with a mordant ,the stain is violet and stains the nuclei satisfactorily.[15,21,22] However, in our case the hibiscus stain was oxidized but not mordanted, yet it appeared red in colour and stained the cytoplasm adequately. The chemical mechanism and the binding effect of the dye to the tissues is still an enigma, yet to be explored and studied, but our study stands novel in a way in using sodium chloride than ferric chloride in an aqueous solution (75%). The staining time used in both cases (Turmeric and Hibiscus) were also novel in our study that is of 120 minutes, which was never used in the earlier studies.

Conclusion

The aqueous stain of turmeric and hibiscus obtained from the rhizomes of Curcuma Longa and the petals of Hibiscus Rosa Sinesis respectively can be used as an alternate to eosin as an cytoplasmic stain. In our study a statistical analysis compared six parameters between hibiscus, turmeric and eosin and it was found that no significant statistical difference was found in terms of nuclear staining, cytoplasmic staining, clarity of staining, uniformity of staining, background staining, thus proving the efficacy of aqueous extract of turmeric and hibiscus as an successful replacement to eosin. However, Eosin proved to be better staining agent in comparison to turmeric and hibiscus in terms of cell morphology.

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Table 1
Extract solution formulation procedure

Eosin stain preparation	01 gram of Eosin is mixed with 80 ml of distilled water, 320 ml of 95% alcohol 0.4 ml of glacial acetic acid.
10% aqueous extract of turmeric	05 grams of rhizomes of turmeric are grounded into a paste using a glass mortar and pestle and mixed with 50 ml of distilled water. The solution was heated on an electric heater for 15 minutes. The solution thus obtained was allowed to cool to room temperature. The solution was filtered on a tripod stand with the help of Whatmanns filter paper no 41. The filtrate thus obtained was used as a stain.
75% aqueous extract of hibiscus	15 grams of Hibiscus petals are weighed using an electric weigh balance, grounded into a paste using a glass mortar and pestle. The grounded mixture was added to 20ml of distilled water. The solution thus prepared was heated on an electric heater for 15 minutes. The solution was allowed to cool to room temperature, filtered on a tripod stand with the help of Whatmanns filter paper no 41. The filtrate thus obtained was used as a stain.0.3 ml of glacial acetic acid and 0.5 grams of sodium chloride were added to this extract.

Table 2
Staining procedure protocol

Procedure		Hematoxylin&Eosin staining	Hematoxylin&10% aqueous extract of turmeric	Hematoxylin and 75% aqueous extract of hibiscus
Deparaffinization	Xylene I	5 min	5 min	5 min
	Xylene II	5 min	5 min	5 min
Rehydration	Absolute Alcohol I	3 min	3 min	3 min
	Absolute Alcohol II	3 min	3 min	3 min
	80% Alcohol	3 min	3 min	3 min
	60% Alcohol	3 min	3 min	3 min
	Water wash	3 min	3 min	3 min
Nuclear staining	Harris' hematoxylin	2 min	2 min	2 min
	Running tap water	30 min	30 min	30 min

Differentiation	1% Acid alcohol	10 sec	10 sec	10 sec
Bluing	Tap water wash	10 min	10 min	10 min
Dehydration	70% Alcohol	2 min	No dehydration is done	No dehydration is done
	80% Alcohol	2 min		
	Absolute Alcohol	2 min		
Cytoplasmic staining		Eosin (1 min)	10% aqueous extract of turmeric (120 min)	75% aqueous extract of hibiscus (120 min)
Dehydration		Absolute Alcohol I &II (3 min)	Absolute alcohol (10 sec)	Absolute alcohol (10 sec)
Clearing		Xylene I & Xylene II (3 min)	Xylene (10 sec)	Xylene (10 sec)
Slide mounted with DPX				

Excess stain were removed by blotting with the help of whatmanns filter paper

Table 3
Criterias used for analysis of tissue sample after staining

Parameters		Eosin Turmeric: Group A (%)	Eosin Hibiscus: Group B (%)	Eosin : Group C (%)	p value			k Value
					A vs B	A vs C	B vs C	
Nuclear staining	0- Inadequate	1.43	7.14	0				
	1- Adequate	98.57	92.86	100	0.09	0.92	0.07	0.86
Cytoplasmic staining	0- Inadequate	8.57	5.71	0				
	1- Adequate	91.43	94.29	100	0.38	0.041*	0.063	0.86
Uniformity of staining	0- Inadequate	2.86	8.57	0				
	1- Adequate	97.14	91.43	100	0.14	0.57	0.041*	0.86
Clarity of staining	0- Inadequate	11.43	21.43	0				
	1- Adequate	88.57	78.57	100	0.11	0.037*	0.06*	0.86
Background staining	0- Absent	84.29	82.86	100				
	1- Present	15.71	17.14	0	0.82	0.013*	0.009*	0.86
Cell morphology	0- Absent	94.29	82.86	0				
	1- Present	5.71	17.14	100	0.04*	<0.01*	<0.01*	0.86

*: statistically significant

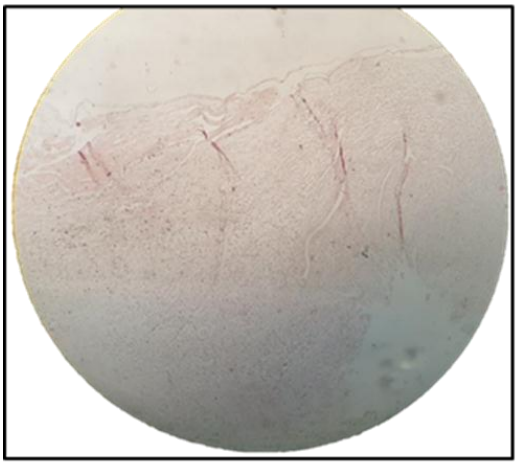

Figure 1. Hibiscus stain depicting clarity of staining under 10 X magnification

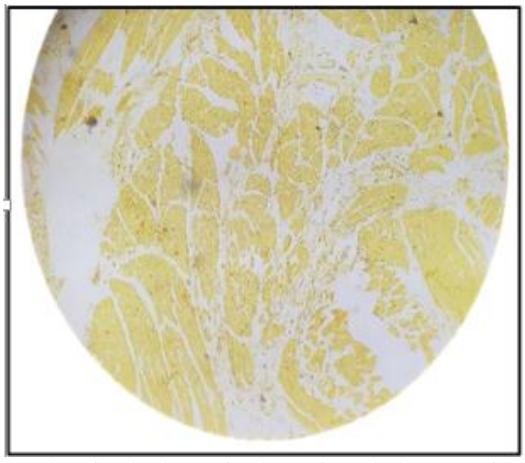

Figure 2. Turmeric stain depicting clarity of staining under 10X magnification

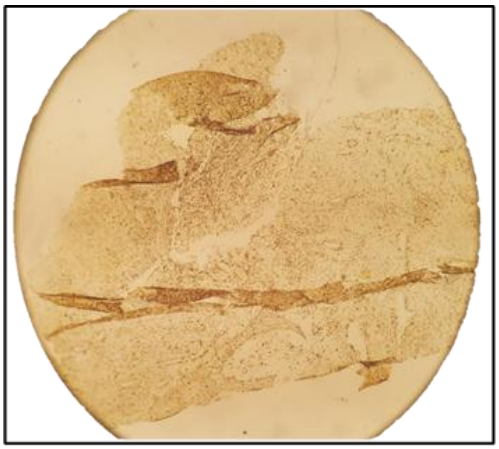

Figure 3. Turmeric staining depicting background staining under 10X magnification

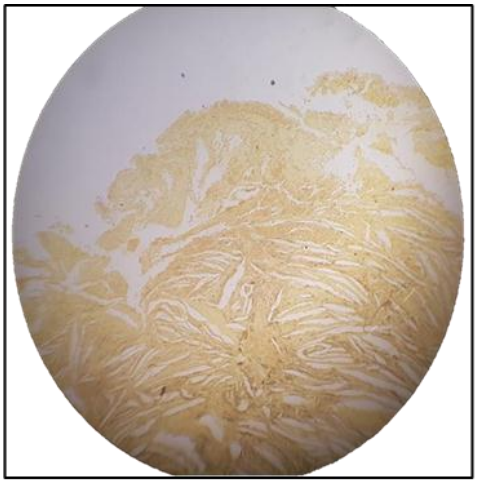

Figure 4. Turmeric stain depicting uniformity of stain under 10 X magnification

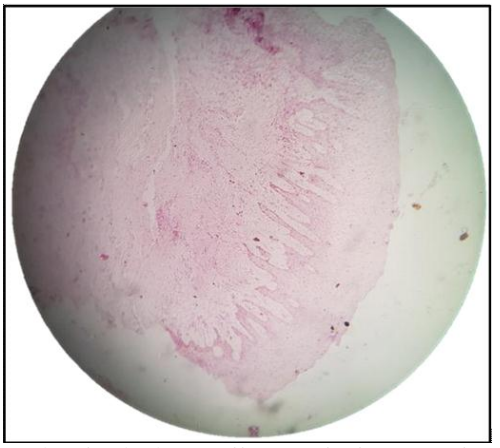

Figure 5. Hibiscus stain depicting cell morphology under 10X magnification

Figure 6. Turmeric staining depicting cytoplasmic staining under 10X magnification