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A comparative cytological study of Eosin & eco-friendly cytoplasmic stains -A Pilot Study

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Abstract:

Background*: Eosin, the widely used synthetic dye is efficient but it is hazardous to human & animal health. So it is advisable to use more eco-friendly, biodegradable & economical alternatives which can be effectively obtained from plants & other natural sources. Turmeric (Curcuma longa), hibiscus (Hibiscus rosa sinensis), heena (Lowsonia inarmis) are the potential natural alternatives for eosin in routine oral cytological procedure. Objective of study is to compare efficacy of turmeric, hibiscus & heena natural stains with synthetic eosin counter stain in oral cytology.*

Materials and Methods*: Method: All the natural dyes were prepared by easy, ready to use methods & used in place of eosin by established protocol techniques for oral cytology in normal and pathological cases. All the slides were coded and were reviewed by two blinded oral pathologist for efficacy and efficiency.*

Results*: It was revealed that turmeric, hibiscus & heena can be used as a counter stain after hematoxylin with varying staining efficacy and efficiency. Inter group comparison (>2 groups) was done using Kruskall Wallis ANOVA followed by pair wise comparison using Mann Whitney U test. Comparison of frequencies of categories of variables with groups was done using chi square test.*

Conclusion: *Easily available & economical turmeric, hibiscus & heena are attractive alternatives to eosin at basic ground level pathology clinics.*

Key Word*: Curcuma, Hibiscus, Heena, Eco-friendly stain*

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I. Inroduction

Nature has lots of wonderful resources for human. Color is one of the elements of nature which made our living more aesthetic and fascinating. Most of the stains used in pathology are chemically synthesized. The advantage of these chemically synthesized stains are good fastness properties, easy availability at economical price and give broad variety of color but they cause skin allergies and other harmful effects to humans, animals, plants and soil[1,2]. Therefore, it is advisable to use more eco-friendly, biodegradable & economical alternatives which can be effectively obtained from plants & other natural sources.

Routine Papanicolaou (PAP) staining is a commonly employed chairside cytological procedure in the diagnosis of suspicious oral smears. Eosin, the widely used synthetic dye is efficient but it is hazardous to human & animal health, for this reason we should use potential natural alternatives for eosin like Turmeric (*Curcuma longa*), heena (*Lowsonia inarmis*), hibiscus (*Hibiscus rosa sinensis*) in routine oral cytological procedures.

Turmeric is also known as Haldi in hindi, It is the dried rhizome powder of C. longa, a perennial herb of the Zingiberaceae (ginger) family, which is 3–5 feet tall bearing oblong, pointed, short- stemmed leaves and funnel- shaped yellow flowers[3].

It contains about 5% diaryl heptanoid colouring materials known as curcuminoids, the chief of which is curcumin (diferuloylmethane) together with smaller quantities of dicaffeoylmethane, caffeoylferuloylmethane

and dihydrocurcumin[4]. It is commonly used as a spice and is also used in the coloring of textiles, drugs, cosmetics, food products[5]. It stains the cytoplasm in a fashion similar to eosin, except for its yellow colour[4].

Heena belongs to Lythraceae family. Henna is a naturally occurring plant widely known as a cosmetic agent used to color hair, skin and palm and also for body art. Heena leaves are the source of red-brown pigment which is called as "lawsone" (2-hydroxy-1, 4-napthaquinone). It has affinity for bonding with protein and also has a fast-dyeing property[6,7].

Hibiscus is a genus of plants belongs to Malvacea family[8]. It is known for their colorful and showy flowers. Hibiscus calyces are rich in anthocyanin, ascorbic acid, and hibiscus acid. The bright color of the Hibiscus calyces is by virtue of the presence of anthocyanin[9]. Flower extracts of hibiscus have been used extensively in the textile industry because of their vibrant and long lasting color. The calyces have also been used as food in food product like jams, juices and in pharmaceutical syrups[10,11].

The present pilot study aimed to compare efficacy of turmeric, hibiscus & heena natural stains with synthetic eosin counter stain in oral cytology and to evaluate and compare nuclear and cytoplasmic details, staining intensity along with clarity of staining of turmeric, heena & hibiscus.

II. Material And Methods

Study Design & Study Area: This was Hospital based pilot study, conducted in the Department of Oral Pathology and Microbiology, RUHS College of Dental Sciences, Jaipur from April-19 to June-19. Informed consent was taken from patients. Ethical clearance was obtained from institutional ethics committee

Sample Size & Sampling methods: 5 male patients, age group 30-40 year who have tobacco habit attending the OPD of our college were taken. After written patient consent, patients were briefed about the procedure. Now 4 oral cytological smears were taken from each patient in the Department of Oral Pathology and Microbiology. The samples were prepared by smearing collected cells on pre marked glass slide, which were immersed in 95% ethanol, fixed, and stained. One smear was stained with Rapid PAP stain and rest 3 slides were stained with Turmeric, Heena & Hibiscus instead of eosin as cytoplasmic stain in cytology.

Inclusion criteria:

1.Patient having tobacco chewing habit
2.Either sex
3.Age- 30-40 years

Exclusion criteria:

1.Pregnant women;
2.Patients with genetic disorders

Methods- Turmeric dye preparation method: Followed by Suryawanshi et al[12].
Heena dye preparation method: Followed by Chukwu et al[13].
Hibiscus dye preparation method: Followed by Sridhara et al[9].

Examination of slides: Based on the quality of staining, the slides were graded as 1, 2, 3 to observe the nuclear details, cytoplasmic details, staining intensity and clarity of staining of turmeric, heena & hibiscus (Table-1).

Table 1: Criteria for grading the stained slides

Quality	Grade
Poor	1
Good	2
Excellent	3

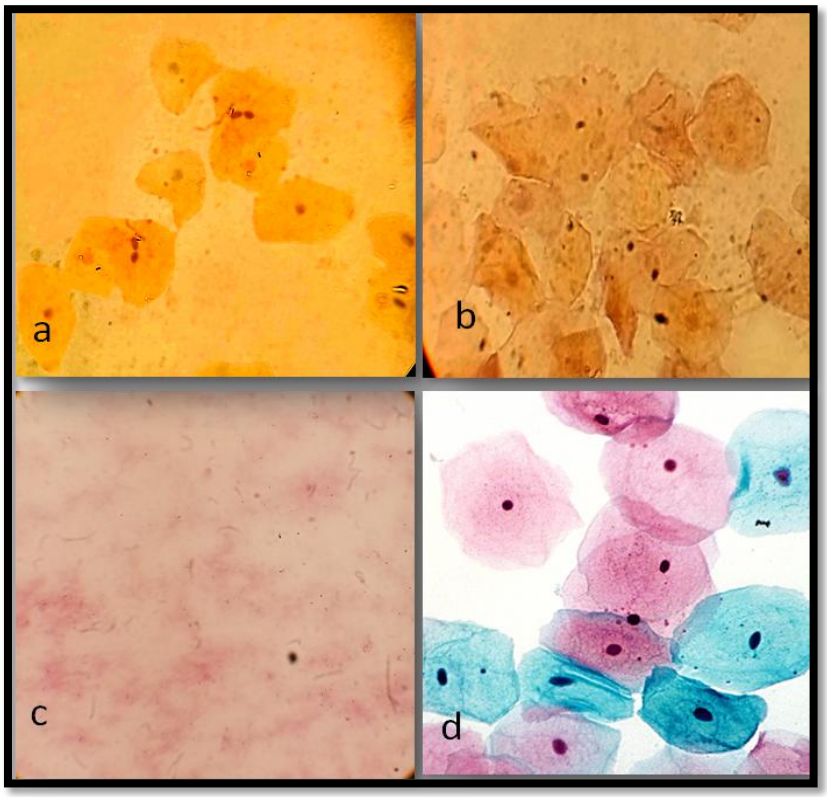

Figure 1: Photomicrograph of oral cytological smear. (a) x40 Haematoxylin & Turmeric (b) x40 Haematoxylin & Heena (c) x10 Haematoxylin & Hibiscus (d) x40 Haematoxylin & Eosine.

Data analysis

- Data obtained was compiled on a MS Office Excel Sheet (v 2019, Microsoft Redmond Campus, Redmond, Washington, United States).
- Data was subjected to statistical analysis using Statistical package for social sciences (SPSS v 26.0, IBM).
- Descriptive statistics like frequencies and percentage for categorical data, Mean & SD for numerical data has been depicted.

  Since the data was codes on a scale, **non-parametric tests** have been used for comparisons.
  - ✓ Inter group comparison (>2 groups) was done using Kruskall Wallis ANOVA followed by pair wise comparison using Mann Whitney U test.
  - ✓ Comparison of frequencies of categories of variables with groups was done using chi square test.

For all the statistical tests, $p<0.05$ was considered to be statistically significant, keeping α error at 5% and β error at 20%, thus giving a power to the study as 80%.

* = statistically significant difference ($p<0.05$)

** = statistically highly significant difference ($p<0.01$)

\# = non significant difference ($p>0.05$) … **for all tables**

III. Result

In this study we found that nuclear detail for turmeric and heena is comparable with eosin, cytoplasmic detail for turmeric and heena is satisfactory and hibiscus as a natural cytoplasmic stains gave poor result for all the staining criteria in this study

Comparison of frequencies of categories of variables
Group * Nuclear detail

Table-2

		Nuclear detail					
		1	2	3	Total	Chi square value	p value
Group	Eosin	0	1	4	5		

	Turmeric	1	2	2	5		
	Heena	0	2	3	5	10.489	0.106#
	Hibiscus	0	5	0	5		
	Total	1	10	9	20		

There was a statistically non significant difference seen (Table-2) for the frequencies between the groups ($p>0.05$).

Group * Cytoplasmic detail

Table-3

		Cytoplasmic detail					
		1	2	3	Total	Chi square value	p value
Group	Eosin	0	1	4	5		
	Turmeric	2	3	0	5		
	Heena	2	3	0	5	18.00	0.006**
	Hibiscus	4	1	0	5		
	Total	8	8	4	20		

There was a statistically highly significant difference seen (Table-3) for the frequencies between the groups ($p<0.01$) with higher freq for score 1 with HIBISCUS, score 2 with HEENA & TURMERIC while score 3 with eosin.

Group * Staining intensity

Table-4

		Staining intensity					
		1	2	3	Total	Chi square value	p value
Group	Eosin	0	2	3	5		
	Turmeric	1	4	0	5		
	Heena	3	2	0	5	16.11	0.013*
	Hibiscus	4	1	0	5		
	Total	8	9	3	20		

There was a statistically significant difference seen (Table-4) for the frequencies between the groups ($p<0.05$) with higher freq for score 1 with HIBISCUS & HEENA, score 2 with TURMERIC while score 3 with eosin.

Group * Clarity

Table-5

		Clarity					
		1	2	3	Total	Chi square value	p value
Group	Eosin	0	1	4	5		
	Turmeric	1	4	0	5		
	Heena	1	4	0	5	26.095	0.000**
	Hibiscus	5	0	0	5		
	Total	7	9	4	20		

There was a statistically highly significant difference seen (Table-5) for the frequencies between the groups ($p<0.01$) with higher freq for score 1 with HIBISCUS, score 2 with HEENA & TURMERIC while score 3 with eosin.

Group comparison of median of scores (n=5 per group)

Table-6

	Group	Mean	Std. Deviation	Median	Chi-Square value	p value of Kruskal Wallis Test
Nuclear detail	Eosin	2.80	.447	3		
	Turmeric	2.20	.837	2		
	Heena	2.60	.548	3	6.098	0.107#
	Hibiscus	2.00	.000	2		
Cytoplasmic detail	Eosin	2.80	.447	3		
	Turmeric	1.60	.548	2	11.294	0.010*
	Heena	1.60	.548	2		
	Hibiscus	1.20	.447	1		
Staining intensity	Eosin	2.60	.548	3		
	Turmeric	1.80	.447	2	10.685	0.014*
	Heena	1.40	.548	1		
	Hibiscus	1.20	.447	1		
Clarity	Eosin	2.80	.447	3		
	Turmeric	1.80	.447	2	14.476	0.002**
	Heena	1.80	.447	2		
	Hibiscus	1.00	.000	1		

There was a statistically highly significant difference seen (Table-6) for the values between the groups ($p<0.01$) for Clarity with higher values in eosin while lower values in Hibiscus.

There was a statistically significant difference seen for (Table-6) the values between the groups ($p<0.05$) for Cytoplasmic detail with higher values in eosin while lower values in Hibiscus

Staining intensity with higher values in eosin while lower values in Hibiscus

There was a statistically non significant difference seen (Table-6) for the values between the groups ($p>0.05$) for Nuclear detail.

IV. Discussion

Acidic structures are stained by basic dyes while basic structures are stained by acidic dyes. The principle of pap stain is to clearly distinguish between basophilic and acidophilic cell components[14]. Nuclear staining, cytoplasmic details and clarity were good in this study with rapid PAP.

Turmeric is valuable because its principle coloring constituent curcumin, which gives yellow color to textile fibers and food[15]**.** The ability of a dye to stain specific tissue structures is determined by certain factors, one of which is the acidity of the stain[4].

Due to the strong affinity of turmeric for the cytoplasm, it can be deduced that the turmeric extract dye is acidic in nature. This deduction is corroborated by the phytochemical analysis of the active column fraction. It contained flavonoids, which are typically polyphenolic compounds. Phenols are acidic, due to their ability to release the hydrogen from their hydroxyl group, hence the ability of turmeric to stain the basic parts of the cell[4].

Turmeric also stains the cytoplasm and gives yellowish orange color. This shows that, the reaction of the turmeric stain is similar to the reaction of eosin in the hematoxylin and eosin technique except for its yellow coloration[4].

This study shows (Figure 1a) nuclear staining was excellent with turmeric & cytoplasmic details, staining intensity and clarity were good similar to one of previously conducted study by Marin Abraham et al[16] in2017 in pathological tissue. On the relative comparison between haematoxylin and eosin and haematoxylin and turmeric, Suryawanshi H et al[12] in 2017, found that the staining ability of epithelium, keratin, muscles, adipocytes, blood vessels and RBCs with curcumin was almost as good as eosin whereas for collagen fibers, cartilage and bone staining ability of curcumin was not as good as eosin in tissue.

The various phytochemical constituents portray the henna plant extracts as a successful potential natural dye. For example in the context of staining, the red colour substance in the extract (tannin) makes the plant a true natural dye. Saponins are also known to reduce surface tension and this property enhances staining[14]. In a previous study which was done by in Adisa JO et al[14] in 2017, heena gave a golden yellow color on liver tissue, but in this study heena gives brown yellow color (Figure 1b) in cytologic smear.

Heena as a cytoplasmic stain in this study shows excellent nuclear detail, good cytoplasmic detail and clarity but staining intensity is poor in contrast to Adisa JO et al[14] in 2017 where Aqueous or ethanolic extracts of henna is acidic and the use of 1% solution of the extract in water, applied for 10min on liver tissue, as a cytoplasmic stain gave excellent result comparable to the conventional haematoxylin and eosin method.

Nuclear detail, cytoplasmic detail, staining intensity and clarity (Figure 1c) were not satisfactory with hibiscus in this study in contrast to previousely done study by Sridhara et al[9] in 2016 found that ethanolic extract of hibiscus showed optimal contrast & staining intensity both in epithelium and connective tissue. They suggest the parallel staining quality of Hibiscus extract with regards to eosin. Literature also suggests the versatility of Hibiscus staining. The extract also has been used as a special stain for parasites, fungi, and neural tissues.

Although a number of studies using ecofriendly stains have been done in past but all remain confined to histology and this study is one of a kind as it is based on cytology. This type of staining can be done on ground level like primary health care centres and give patient a reality check about the status of suspected lesion in very less time. This study also helps to check the feasibility of these ecofriendly staning so we can use them when eosin is not available.

V. Conclusion

Turmeric & Heena are cost effective, readily available, natural & safe alternative to eosin which is a relatively more expensive synthetic dye. Hibiscus is also a natural dye but in this study the result was not satisfactory so further studies are required. Still the gold standard in oral cytology is EOSIN.

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