Journal Homepage: - www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)

Article DOI: 10.21474/IJAR01/17161
DOI URL: http://dx.doi.org/10.21474/IJAR01/17161

RESEARCH ARTICLE

"COMPARATIVE EVALUATION OF THE EFFICACY OF FIXATIVE AGENTS AND DIFFERENT STAINS FOR EVALUATING MAST CELLS MANUALLY AND DIGITALLY"

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Manuscript Info

Manuscript History
Received: 26 April 2023
Final Accepted: 31 May 2023
Published: June 2023

Abstract

Introduction- Mediators of inflammation are generated by mast cells. According to research articles, mast cell identification is based on the differential staining of secretory granules but it is always not possible to distinguish reliably between mast cells and basophils in tissues.[1] Very few study are done till date to assess the Oral lesions using photometric analysis. So in the present study, RGB was used to compare the efficacy of Stains with the aid of computerised photometric analysis using RGB scoring.

Aim - To evaluate and compare the efficacy of fixative agents (10% Neutral Buffered Formalin and Absolute Methanol) and stains (Hematoxylin and Eosin, Toluidine blue and May Grunwald Giemsa stain) for intensity of stain to identify mast cells manually under Light microscope by three independent observers and digitally by Photo Processing software using RGB values in Adobe Photoshop 7.0.

Materials and methods - 100 randomly selected Oral Lichen Planus cases were diagnosed using WHO criteria for histopathological examination. Samples were collected by doing punch biopsy and divided into 2 study groups. **Group A** – 50 tissue samples were fixed in 10% Neutral buffered Formalin for minimum period of 24 hours. **Group B** – 50 tissue sections fixed in Absolute Methanol for 15 min.

Tissues from both the groups were further divided into three parts and stained with H&E, Toluidine blue and MGG stains respectively. The mounted sections of both Group A and Group B were evaluated on the basis of intensity of stain for identification of mast cells by three independent observers under light microscope and digitally using Adobe Photoshop version 7.0 using RGB values for each stained slide respectively and data was statistically analysed.

Results - Present study shows non-significant difference in intensity of stain in Group A and Group B between Toluidine Blue and MGG stain whereas difference between Toluidine Blue and H&E stain, MGG and H&E stain was statistically significant for manual observations obtained by three independent observers. Evaluation of intensity of color based on RGB values shows statistically significant difference for Red and Blue color on H&E stained sections. For Toluidine Blue and MGG stained sections, there is statistically significant difference in the

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color intensity of Red, Green and Blue color with p value less than 0.005**.**In Group A there was non-significant difference in intensity of Green color between H&E and MGG stain while statistically Significant difference for red, green and blue color between Toluidine Blue, MGG and H&E stain with p value less than 0.005.In Group B, statistically significant difference in the color intensity of Red, Green and Blue color for H&E, Toluidine blue and MGG stain was observed.

Conclusion –It was found that

1. Absolute Methanol is more effective in fixation as compared to 10% Neutral buffered formalin which is otherwise the gold standard on account of its effectiveness, low cost and consistent results to identify the mast cells.
2. The efficacy of H&E stain is the lowest among H&E, Toluidine blue and MGG stain to identify the mast cells.
3. The intensity of Red, Green and Blue color shows good contrast among H&E, Toluidine blue and MGG stain when fixed with Absolute Methanol as compared when fixation is done with 10% Neutral Buffered Formalin to identify the mast cells.

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Introduction:-

"Mastzellan"—a well fed cell, name given by **Paul Ehrlich**who discovered granular cell in loose connective tissue in 1877**.**He explained that mast cells are also associated with inflammation. Thus, mast cells are considered as potent effector cells of the immune system. Mast cells have important role in allergic diseases, anaphylaxis, autoimmunity, reproductive disorders. However, still research is going on its role in etiopathogenesis of oral lesions.[2]

Mediators of inflammation are generated by mast cells in form of granules which on activation, secreted either by allergen crosslinking of membrane-bound IgE or through other stimuli. According to research articles, mast cell identification is based on the differential staining of secretory granules but it is always not possible to distinguish reliably between mast cells and basophils in tissues.[1] Mast cells are spindle to oval shaped and exhibit the same staining characteristics as fibroblasts with Hematoxylin and Eosin (H&E); therefore, mast cells can be difficult to distinguish from fibroblasts **(Ankle et al. 2007)[3].**

Materials And Methods:-

Sample selection –

In this study, 100 randomly selected Oral Lichen Planus cases referred from the Oral Medicine department were diagnosed using World Health Organization criteria for histopathological examination.

Samples were collected by doing punch biopsy in the Department of Oral and Maxillofacial Pathology and Microbiology, Divya Jyoti College of Dental Sciences & Research, Modinagar for histopathological diagnosis.

This tissue sections were further divided into 2 study groups.

Group A – Immediately following biopsy, 50 tissue samples were placed in 10% Neutral buffered Formalin (Sigma- Aldrich) for both preservation and fixation for minimum period of 24 hours.

Group B – immediately following biopsy, 50 tissue sections fixed in Absolute Methanol (Sigma- Aldrich) for both preservation and fixation for 15 min.

Tissues from both the groups i.e. Group A and Group B were further divided into three parts and stained with H&E(Nice Chemical Pvt Ltd.), Toluidine blue (India mart)and MGG (Anamol) stains respectively.

H&E staining –
Deparaffinization done and slides were hydrated through 100, 90 and 70% alcohol and washed with distilled water. Slides were dipped in Harris' hematoxylin for 5 min, then differentiated by dipping the slide in 1% acid alcohol for 1 min, washing in tap water for 7−8 min for bluing, dipping in eosin for 1−2 sec and washing in tap water. Slides were dehydrated through increasing grades of alcohol for 5 min each, cleared in xylene and mounted with DPX.[4]

Toluidine blue staining –
Deparaffinization done and slides were hydrated through 100, 90 and 70% alcohol and washed with distilled water. The slides then were stained with toluidine blue for 3 min, washed in three changes of distilled water, dehydrated quickly in95% alcohol, washed in 100% alcohol, cleared in xyleneand mounted with DPX.[1]

MGG staining –
Deparaffinization done and slides were hydrated through a series of alcohols and washed with distilled water. The slides were placed in May-Grunwald solution for 25 min, washed in tap water,then stained with Giemsa solution for 20 min.After washing in tap water, slides were passed through increasing grades of alcohol for 1 sec each, cleared in xylene and mounted with DPX.[5]

Evaluation of staining –
Each stained slides were evaluated by three oral pathologists as independent observer under light microscope for presence or absence of mast cells, and the intensity of staining was assessed by contrast with surrounding connective tissue.Staining intensity was scored as Grade 1 - mild/poor; Grade 2- moderate; Grade 3- strong/good.[16]

Photographs were transferred to computer terminal and evaluation of intensity of stain was done digitally by Adobe Photoshop Version 7.0 using RGB values shown by histogram for Red, Green and Blue color.(Fig 1-12)

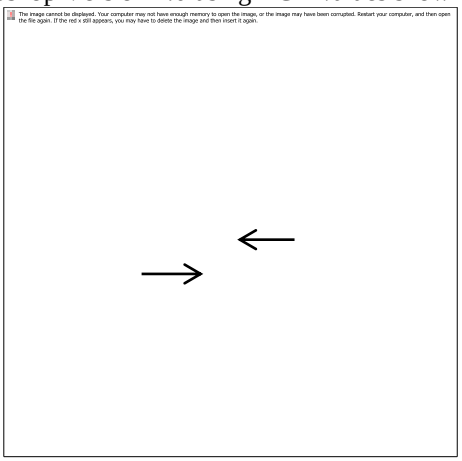

Fig 1:- 10% Neutral buffered Formalin fixed tissue stained with H&E.

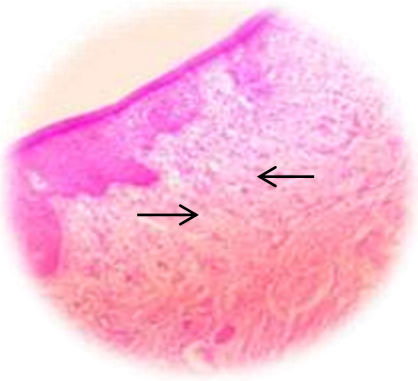

Fig 2:- Absolute Methanol fixed tissue stained with H&E.

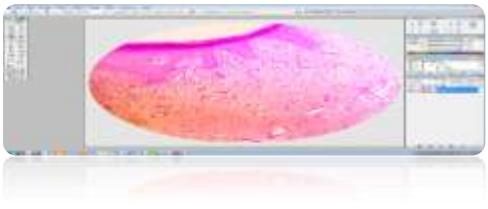

Fig 3:- RGB analysis of tissue fixed with 10% Neutral buffered Formalin and stained with H&E.

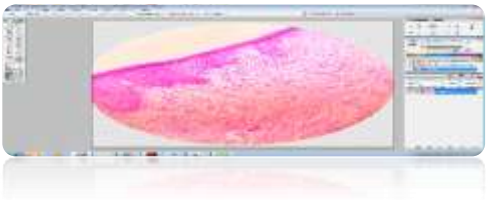

Fig 4:- RGB analysis of tissue fixed with Absolute Methanol and stained with H&E.

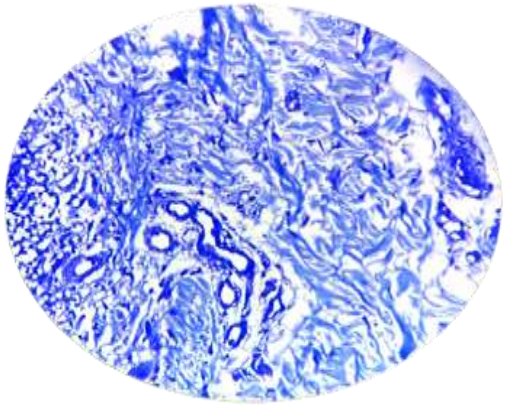

Fig 5:- 10% Neutral buffered Formalin fixed tissue stained with Toluidine blue.

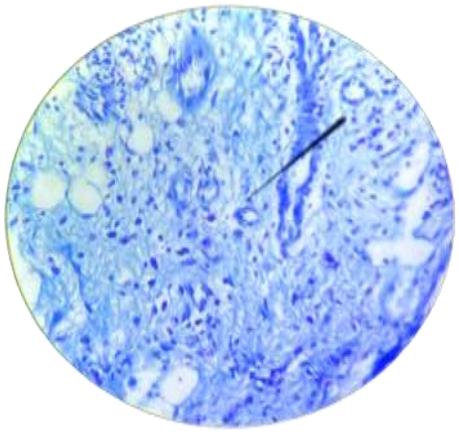

Fig 6:- Absolute Methanol fixed tissue stained with Toluidine blue.

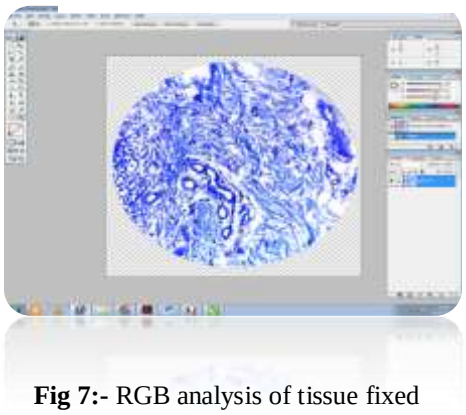

Fig 7:- RGB analysis of tissue fixed with 10% Neutral buffered Formalin and stained with Toluidine blue.

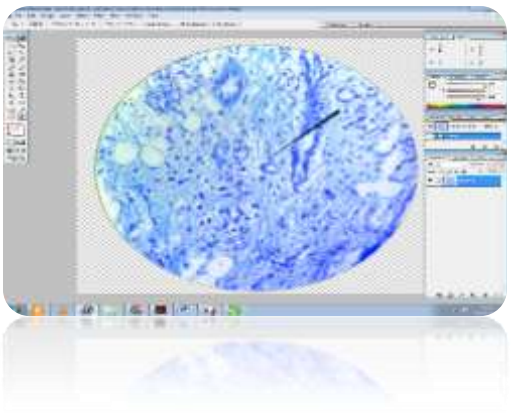

Fig 8 - RGB analysis of tissue fixed with Absolute Methanol and stained with Toluidine blue.

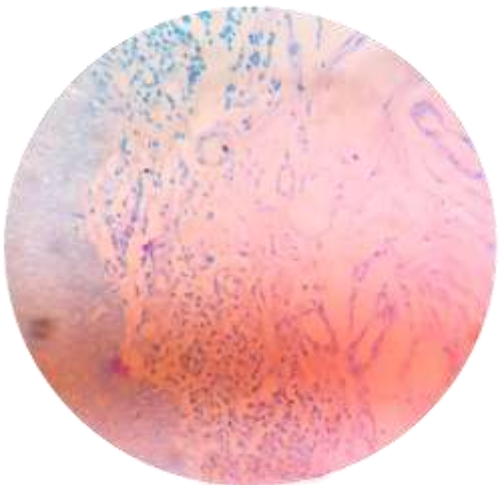

Fig 9:- 10% Neutral buffered Formalin fixed tissue stained with MGG.

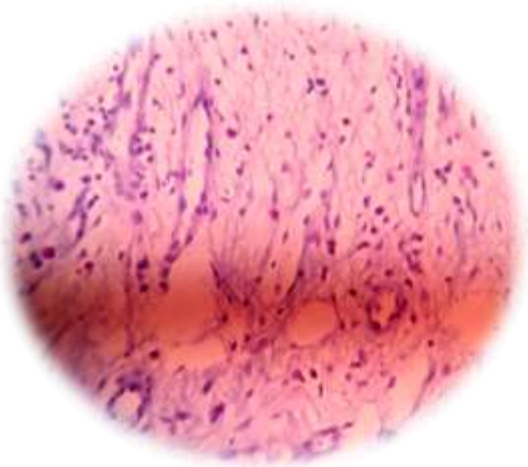

Fig 10:- Absolute Methanol fixed tissue stained with MGG.

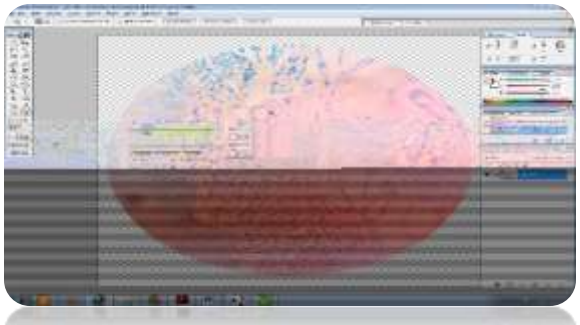

Fig 11:- RGB analysis of tissue fixed with 10% Neutral buffered Formalin and stained with MGG.

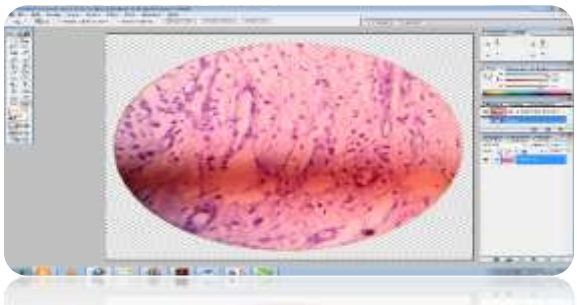

Fig 12:- RGB analysis of tissue fixed with Absolute Methanol and stained with MGG.

Statistical Analysis –

The observations for each slide were tabulated. Mean for each value was obtained. Results were statistically analysed. Comparisons between the Group was performed by Student t-test variable. Comparison of stains within a Group for observations obtained manually under light microscope was performed by ANOVA-F test and Post Hoc analysis was done for intergroup comparison of stains for the RGB values obtained digitally by Adobe photoshop version 7.0 software.

Results:-

The data was collected in a systematic manner and formulated as tables and graphs for interpretation of results.In present study, for H&E stain**,** Toluidine blue stainand MGG stain**(Table 1)** the application of Student T test shows significant difference in intensity of stain between Group A and Group B**..**One way ANOVA-F test revealed non-significant difference between Toluidine Blue and MGG stain whereas difference between Toluidine Blue and H&E stain, MGG and H&E stain was statistically significant in both Group Aand Group B**. (Table 2, Table 2A & Table 2B).**

For RGB values, Student T test shows statistically significant difference in the color intensity of Red and Blue color but statistically not significant for green color among Group A and Group B for H&E stained sections. **(Table 3)**forToluidine Blue and MGG stained sections intensity of stain among Group A and Group B shows statistically significant difference in the color intensity of Red, Green and Blue color**. (Table 4); (Table 5)**.

One way ANOVA testin Group A shows non-significant difference for intensity of Green color between H&E and MGG stain.**(Table 6A &Table 6B).** In Group B, there is statistically significant difference observed in the color intensity of Red, Green and Blue color for H&E stain, Toluidine blue stain and MGG stain when analyzed using One Way ANOVA test. **(Table 7A and Table 7B).**

Table 1:- Mean value for intensity of stain in Group A and Group B for H&E, Toluidine blue and MGG stained sections observed manually by three independent observers.

	H & E Stain		Toludine Blue Stain		MGG Stain	
	Group A	**Group B**	**Group A**	**Group B**	**Group A**	**Group B**
Mean	**0.34**	**0.70**	**0.70**	**0.76**	**1.00**	**1.30**
SD	**0.47**	**0.46**	**0.34**	**0.59**	**0.78**	**0.73**

T Value	**9.465**	**4.129**	**4.162**
P Value	**0.001**	**0.041**	**0.042**
Significance	**Significant**	**Significant**	**Significant**

Table 2:- The mean value for intensity of stain between Toluidine Blue, MGG and H&E stained sections in Group A and Group B observed by three independent observers.

	Mean		STD	
	Group A	**Group B**	**Group A**	**Group B**
H & E	**0.34**	**0.70**	**0.47**	**0.46**
Toludine Blue	**0.70**	**0.76**	**0.34**	**0.59**
MGG	**1.00**	**1.30**	**0.78**	**0.73**

Table 2A:- One way ANOVA-F table for testing the significance in intensity of stain due to groups observed manually by three independent observers.

S.no.	Sum of Squares	Degree of freedom	Mean sum of Square	F- ratio	Sig.
Between Groups	2.184	2	1.092	3.467	0.046
Within Groups	8.504	27	0.315		
Total	10.688	29			

Table 2B:-One way ANOVA-F table for testing the significance in intensity of stain due to groupsgraded by three independent observers.

	Sum of Squares	Degree of freedom	Mean sum of Squares	F-ratio	Sig.
Between Groups	2.184	2	1.092	3.998	0.049 (Sig)
Within Groups	9.833	27	0.364		
Total	12.013	29			

Table 3:- Mean value for intensity of stain in each Group A and Group B for H&E stained sections observed digitally using RGB values.

		Mean	SD	Std Error	P value	Significance
Red	**Methanol**	251.93	2.62	0.117	0.001	Significant
	Formalin	225.65	78.402	3.516		
Green	**Methanol**	140.47	30.041	1.347	0.443	Non- Significant
	Formalin	143.55	84.036	3.769		
Blue	**Methanol**	201.29	39.449	1.769	0.001	Significant
	Formalin	172.48	77.676	3.484		

Table 4:- Mean value for intensity of stain in each Group A and Group B for Toluidine Blue stained sections observed digitally using RGB values.

Color	Intergroup Comparison	Mean	Standard Deviation	Standard Error	P value	Significance
Red (R)	Methanol	125.19	6.69	0.300	0.001	Significant
	Formalin	92.68	27.75	1.245		
Green (G)	Methanol	103.66	11.56	.518	0.001	Significant
	Formalin	97.75	31.94	1.43		
Blue (B)	Methanol	166.07	16.89	0.757	0.001	Significant
	Formalin	236.40	28.01	1.256		

Table 5:- The mean value for intensity of stain in each Group A and Group B for MGG stained sections observed digitally using RGB value.

.		Mean	SD	Std Error	P value	Significance
Red	**Methanol**	173.65	14.262	0.639	0.001	Significant
	Formalin	193.26	47.544	2.132		
Green	**Methanol**	118.80	19.530	0.876	0.001	Significant
	Formalin	146.58	57.493	2.578		
Blue	**Methanol**	186.75	17.831	0.800	0.001	Significant
	Formalin	280.57	45.144	2.025		

Table 6A:- The mean value for intensity of stain in Group A between Toluidine Blue, MGG and H&E stained sections observed digitally using RGB values.

		Mean	SD	Std Error	P value	Significance
	Toluidine Blue Stain	92.683	27.759	1.245		
Red	**MGG stain**	193,26	47.544	2.132	0.001	Significant
	H &E stain	225.65	78.402	3.516		
	Toluidine Blue Stain	97.75	31.940	1.432		
Green	**MGG stain**	146.58	57.493	2.578	0.001	Significant
	H &E stain	143.55	84.036	3.769		
	Toluidine Blue Stain	236.40	28.01	1.256		
Blue	**MGG stain**	280.57	45.144	2.025	0.001	Significant
	H &E stain	172.48	77.676	3.484		

Table 6B:-Post Hoc Analysis for the intensity of stain in Group A between Toluidine Blue, MGG and H&E stained sections graded digitally using RGB values.

Intergroup Comparison		Red	Green	Blue
Toluidine Blue Stain	MGG stain	P=0.001 (Sig)	P=0.001 (Sig)	P=0.001 (Sig)
Toluidine Blue Stain	H &E stain	P=0.001 (Sig)	P=0.001 (Sig)	P=0.001 (Sig)
MGG stain	H &E stain	P=0.001 (Sig)	P=0.438 (Non-Sig)	P=0.001 (Sig)

Table 7A:-The mean value of intensity of stain in Group B between Toluidine Blue, MGG and H&E stained sections observed digitally using RGB values.

		Mean	SD	Std Error	P value	Significance
Red	**Toluidine Blue Stain**	125.19	6.697	.300	0.001	Significant
	MGG stain	173.65	14.262	.639		
	H &E stain	251.93	2.621	.117		
Green	**Toluidine Blue Stain**	103.66	11.561	.518	0.001	Significant
	MGG stain	118.80	19.530	.876		
	H &E stain	140.47	30.041	1.347		
Blue	**Toluidine Blue Stain**	166.07	16.894	.757	0.001	Significant
	MGG stain	186.75	17.836	.800		
	H &E stain	201.29	39.449	1.769		

Table 7B:- Post Hoc Analysis in Group B for intensity of stain between Toluidine Blue, MGG and H&E stain observed digitally using RGB values.

Intergroup Comparison		Red	Green	Violet
Toluidine Blue Stain	MGG stain	P=0.001 (Sig)	P=0.001 (Sig)	P=0.001 (Sig)
Toluidine Blue Stain	H &E stain	P=0.001 (Sig)	P=0.001 (Sig)	P=0.001 (Sig)
MGG stain	H &E stain	P=0.001 (Sig)	P=0.001 (Sig)	P=0.001 (Sig)

Discussion:-

According to **Ankle et al 2007[3]**, mast cells are spindle to oval shaped and exhibit the same staining characteristics as fibroblasts with Hematoxylin and Eosin (H&E); so are undistinguishable from fibroblasts. Similar findings consistent with our study were noted in several other studies conducted by various authors**L. J.Walsh 1990[6], R. Sharma 2010[2], Ramalingam S et al 2018[7], Santosh R et al 2012[8](Table 1).**

Metachromatic granules show differential staining characteristics in mast cells and therefore aids in cell identification using various histochemical stains **(Sharma and Saxena 2011[2]; Singh et al. 2015[9].**

Toluidine Blue proves high affinity for the metachromatic granules present in mast cells so is used to specifically recognize these cells in the study samples.[7]It has been reported that toluidine blue can be used to identify mast cells in oral lesions mainly due to its contrast with background connective tissue **(Sharma and Saxena 2011[2]; Mansata et al. 2014[10]). (Table 1).**

In our study,the efficacy of H&E stain found to be the lowest among H&E, Toluidine blue and MGG stain to identify the mast cells.**Mutsaddi S 2019[11]**reported that MGG provided good contrast between mast cells and background connective tissue, followed by Toluidine blue, Astra blue and ABPY. High contrast facilitates identification of the mast cells.**Leclere et al. 2006[5]** reported that MGG and Toluidine blue stained mast cells comparably, it was reported that toluidine blue and MGG exhibited good agreement for staining of mast cells. It appears that Toluidine blue and MGG stains are equally good for detecting mast cells**.(Table 1)**

Our results revealed that after formaldehyde fixation, few cells took up the stains. In contrast, several mast cells were stained after methanol fixation. These findings are in accord with previous studies in which similar results were observed in studies by **Flint KCet al 1985[12]&Haslam PL et al 1987[13]. (Table 2, Table 2A, Table 2B).**

The RGB color model is additive in the sense that the three light beams are added together, and their light spectra add, wavelength for wavelength, to make the final color's spectrum.[14]Very few study are done till date to assess the Oral lesions using photometric analysis. So in the present study, RGB was used to compare the efficacy of Stains with the aid of computerised photometric analysis using RGB scoring.

Similar results were seen in study conducted by **AbhikSikdar et al 2020[15]**, were efficacy was evaluated using Computerised Photometric Analysis and Red Green Blue (RGB) scoring in the treatment of erosive Lichen Planus.**(Table 3); (Table 4); (Table 5)**; **(Table 6A &Table 6B); (Table 7A and Table 7B).**

Conclusion:-

1. Absolute Methanol found to be more effective in fixation as compared to 10% Neutral buffered formalin which is otherwise the gold standard on account of its effectiveness, low cost and consistent results to identify the mast cells.
2. The efficacy of H&E stain found to be the lowest among H&E, Toluidine blue and MGG stain to identify the mast cells.
3. The intensity of Red, Green and Blue color shows good contrast among H&E, Toluidine blue and MGG stain when fixed with Absolute Methanol as compared when fixation is done with 10% Neutral Buffered Formalin to identify the mast cells.

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