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Original Article

Pathology Section

Efficacy of *Kumkum* as a Surrogate for Eosin in Routine Histological Sections: An Observational Study

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ABSTRACT

Introduction: Stains are a crucial component of laboratory procedure. They help in highlighting the different tissues, both normal and abnormal, which plays an important role in diagnostic histopathology. Synthetic dyes are utilised for majority of the stains in histology. Among the natural surrogates studied, *Kumkum* is less researched.

Aim: To compare the staining characteristics of *Kumkum*, which imparts red colour as an alternative to eosin.

Materials and Methods: This observational study was conducted at Histopathology section, Pathology department at Chettinad Hospital and Research Institute for a period of six months from January 2022 to June 2022. In 50 tissue blocks, each was made into two sections and one was stained with routine Haematoxylin and Eosin (H&E), while the other was stained with Haematoxylin and *Kumkum* solution (H&K). The Cellular Architecture (CA) (based on distinct or indistinct nucleus, cytoplasm) and quality of staining (poor, satisfactory or good) of H&E and H&K solution were compared and a scoring system was given. The scores were analysed with Chi-square test using SPSS software (version 25).

Results: Out of 100 slides, 45 (90%) stained with H&E and 48 (96%) with H&K showed distinct CA. A total of 47 slides (94%) stained with H&K and 41 (82%) with H&E showed good quality of staining.

Conclusion: *Kumkum* solution appears to be an efficient counterstain in place of eosin in highlighting the normal structures in histopathological sections. It highlighted the RBCs in the arteries and cytoplasm of the cells in glandular and squamous epithelium better than or equally good as eosin.

Keywords: Counterstain, Maceration, Staining, Utility of *Kumkum*

INTRODUCTION

Histology or histopathology is the microscopic examination of tissues and cells, even plants and animals. It is done by process of fixing, sectioning, staining and viewing under a microscope. Diagnostic histopathology is an important step in the management of the patient [1]. Staining is useful for microscopy technique and employed to improve contrast in the material, often at the microscopic level. Stains and dyes are often used in biological tissues to highlight structure. Stains can be classified as acidic, basic, or neutral. The colour which is produced depends on the chemical nature of the stains and the cellular constituents. The staining method facilitates diagnosis by increasing the visibility of cells under a microscope. Biological dyes are classified into two categories; natural and artificial. Natural dyes are taken from natural resource; the most dominant natural dye in the histopathology laboratory is haematoxylin. Artificial dyes are derived from chemical agents [2].

H&E stain is the amalgamation of two histological stains. Haematoxylin, a basic dye is from natural dyes obtained from barks of *Haematoxylon campechianum* and also extract from the logwood tree, stains nucleus giving it a bluish-purple colour [3]. Eosin is one of the acidic florescent chemical compounds that binds by basic or eosinophilic compounds and stains cytoplasm, pink to orange and nuclei in dark purple colour. It is one of the commonest counterstain used in routine histopathological sections [3].

Recently, researchers are debating that use of natural substances like beetroot, saffron, rose and turmeric in staining tissues [4,5]. In natural substances, *Kumkum* is one of the compounds that can be surrogative for eosin as it imparts red to dark pink colour to the tissues. The preparation of *Kumkum* powder can be by both commercial method as well as natural method. Mainly, *Kumkum* is taken from saffron flower and pinch of turmeric is added in the commercially available sachets. *Kumkum* is an eco-friendly compound, low cost, readily available, non toxic and organic stain [6].

To our knowledge, only few studies are available exploring *Kumkum* solution utilities in staining the different tissues in histopathology. Lavanya A et al., concluded the utility of dual staining property of *kumkum* for the differentiation of the components of tooth, bone and soft tissue structures in histostaining of oral tissues which facilitates the diagnosis of fibro-osseous lesions, bony, collagen and muscular pathologies [6]. Navya N et al., concluded that *Kumkum* appears to be an efficient counterstain for demonstrating various structures in histopathology sections of cervix tissue [7]. The current study is an attempt to compare the staining characteristics of eosin and Kumkum and prove that Kumkum is the best alternative to eosin. The present study highlights the novel use of *Kumkum* solution in the field of histopathology for the purpose of staining.

MATERIALS AND METHODS

The observational study was conducted at Histopathology section-Pathology Department, Chettinad Hospital and Research Institute, from January 2022 to June 2022. The institutional human ethics committee (IHEC-I/0573/22) reviewed the proposal and approved this study. A total of 50 tissue sections were analysed and used for staining which included normal cervix, endometrium, ovaries, artery, smooth muscle and nerve bundles. From each tissue block two sections were taken in which one was stained with routine H&E and other with H&K (*Kumkum*) stain. A total of 100 slides were used in two groups and the CA and staining efficacy was compared by two pathologists independently and blinded.

Inclusion criteria: Normal and well-fixed cervix, endometrium, ovaries, artery, smooth muscle, skeletal muscle and nerve bundles.

Exclusion criteria: Biopsies that were not processed, not fixed properly and with inadequate tissue structure were not included, malignant neoplasms were not included.

Preparation of Kumkum Solution

The maceration technique was used to prepare *Kumkum* solution. Fifteen grams of commercial *Kumkum* was diluted (Gopuram brand) with 100 mL propan-2-ol. The solutions are kept undisturbed for 48 hours. Filtered solution was used for staining, and the filtrates from those filters were kept in bottles with labels [Table/Fig-1].

[Table/Fig-1]: Storage of *kumkum* solution for 48 hours undisturbed in bottle.

Two sections of formalin-fixed paraffin embedded tissue measuring 5 µm thick were cut. The routine H&E staining was used in the first set. The prepared *Kumkum* stain was used for the second set of slides. Every batch was verified by using quality control slide and the timing of *Kumkum* solution was standardised to 4-5 minutes by trial and error method. A pH metre was used to calculate pH levels. A pH level of 5.45-5.52 was maintained in *Kumkum* solution. Two pathologists who were blinded independently evaluated the staining characteristics of both the groups using a scoring system [8]:

1. Cellular architecture (CA):
   Score-0: Indistinct nucleus- cytoplasm
   Score-1: Distinct Nucleus- cytoplasm
2. Quality of Staining (QS)
   Score-0=Poor
   Score-1=Satisfactory
   Score-2=Good

Steps in staining of Haematoxylin and *Kumkum* (H&K):

- Deparaffinise section followed by three changes of xylene five minutes each.
- Re-hydrate in absolute alcohol for five minutes.
- 70% alcohol for two minutes.
- Wash in running tap water for five minutes.
- Haematoxylin solution for eight minutes.
- Wash in running tap water for three minutes.
- 1% Acid alcohol one dip.
- Wash in running tap water for three minutes.
- 1% Lithium carbonate.
- Wash in running water for five minutes.
- Kumkum solution for four minutes.
- Rinse in 95% alcohol- 10 dips.
- Blot and mount.

STATISTICAL ANALYSIS

Results were tabulated and statistically analysed using chi-square test using SSPSS software (version 25).

RESULTS

Out of 100 slides, 45 (90%) stained with H&E and 48 (96%) with H&K, showed distinct CA. A total of 47 slides (94%) stained with H&K and 41 (82%) with H&E showed good quality of staining [Table/Fig-2].

Parameters	Score-0	Score-1	Score-2
H&K (CA)	2 (4%)	48 (96%)	NA
H&E (CA)	5 (10%)	45 (90%)	NA
H&K (QS)	1 (2%)	2 (4%)	47 (94%)
H&E (QS)	2 (4%)	7 (14%)	41 (82%)

[Table/Fig-2]: Comparision of scores of Cellular Architecture (CA) and Quality of Staining (QS) by H&K and H&E.

By crosstabulation of scores obtained from scoring of CA by Observer-1 and Observer-2 in H&E [Table/Fig-3] and H&K slides [Table/Fig-4], it was found that the Kappa value was 0.540 and 0.658 respectively, p-value was 0.001 both of which found to be statistically significant. Similarly, cross tabulating the scores obtained from scoring of quality of staining by Observer-1 and Observer-2 in H&E [Table/Fig-5] and H&K slides [Table/Fig-6], it was found that the Kappa value was 1.000 and 0.826, respectively, p-value of 0.001 both of which were found to be statistically significant.

Scoring of Cellular Architecture (CA) in H&E		Obs2		
		Indistinct nucleus-cytoplasm	Distinct Nucleus-cytoplasm	Total
Obs1	Indistinct nucleus-cytoplasm	2	2	4
	Distinct Nucleus-cytoplasm	1	45	46
Total		3	47	50
Measure of agreement		Kappa value	Approximate T[b]	Approximate significance
		0.540	3.863	0.001

[Table/Fig-3]: Scoring of Cellular Architecture (CA) in Hematoxylin and Eosin (H&E) by Observer-1 (Obs1) and scoring of Cellular Architecture (CA) in H&E by Observer-2 (Obs2) Crosstabulation.
Note: b. Using the asymptotic standard error assuming the null hypothesis

Scoring of Cellular Architecture (CA) in H&K		Obs2		
		Indistinct nucleus-cytoplasm	Distinct nucleus-cytoplasm	Total
Obs1	Indistinct nucleus-cytoplasm	1	0	1
	Distinct nucleus-cytoplasm	1	48	49
Total		2	48	50
Measure of agreement		Kappa value	Approximate T[b]	Approximate significance
		0.658	4.949	0.0001

[Table/Fig-4]: Scoring of Cellular Architecture (CA) in Haematoxylin and Kumkum (H&K) by Observer-1 (Obs1) and scoring of CA in H & K by Observer-2 (Obs2) Crosstabulation.
Note: b. Using the asymptotic standard error assuming the null hypothesis

Scoring of quality of staining in H&E		Obs2			
		Poor	Satisfactory	Good	Total
Obs1	Poor	1	1	0	2
	Satisfactory	0	1	0	1
	Good	0	0	47	47
Total		1	2	47	50
Measure of agreement		Kappa value	Approximate T[b]	Approximate significance	
		1.000	8.412	0.0001	

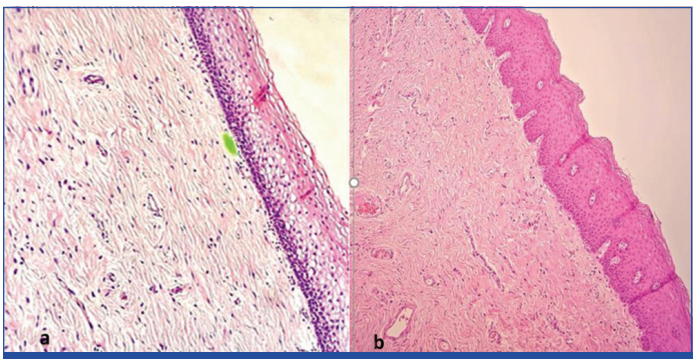
[Table/Fig-5]: Scoring of quality of staining in H&E by Observer-1 (Obs1) and scoring of quality of staining in H&E by Observer-2 (Obs2) crosstabulation.
Note: b. Using the asymptotic standard error assuming the null hypothesis

Staining Characteristics of Different Cells of Kumkum

Squamous epithelium: The ectocervix lined by squamous epithelium was taken to compare. *Kumkum* staining highlighted all the layers of the epithelium clearly as eosin [Table/Fig-7].

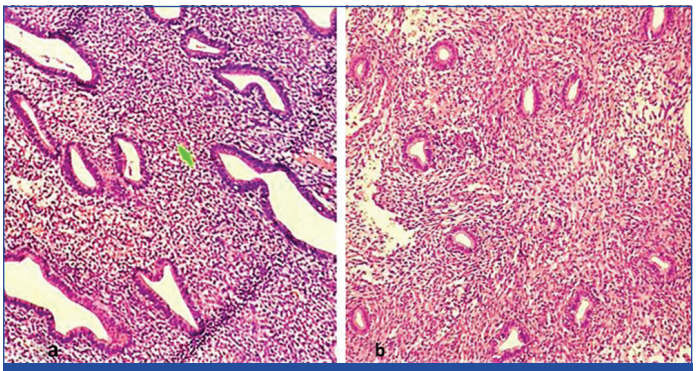
Scoring of quality of staining in H and K		Obs2			Total
		Poor	Satisfactory	Good	
Obs1	Poor	1	1	0	2
	Satisfactory	0	1	0	1
	Good	0	0	47	47
Total		1	2	47	50
Measure of agreement		Kappa value	Approximate T[b]	Approximate significance	
		0.826	7.733	0.0001	

[Table/Fig-6]: Scoring of quality of staining in H&K by Observer 1 (Obs1) and scoring of quality of staining in H & K by Observer 2 (Obs2) Crosstabulation.
Note: b. Using the asymptotic standard error assuming the null hypothesis



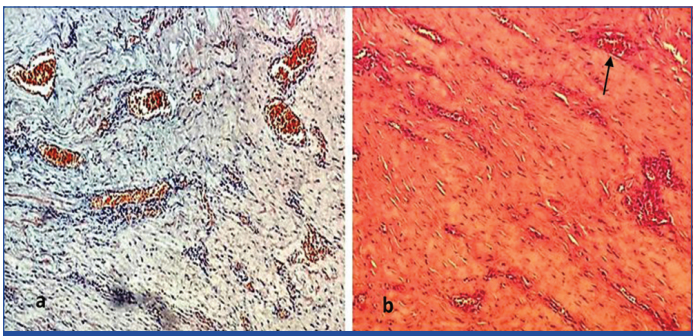
[Table/Fig-7]: a) Photographs showing ectocervix with squamous epithelium (H&K, 20X); b) (H&E, 20X).

Glandular epithelium: The long and tubular endometrial glands took the bright stain with blue nucleus. At places the subnuclear vacuolation can also be appreciated well [Table/Fig-8].



[Table/Fig-8]: a) Photographs showing endometrial glands with stroma (H&K, 20X); b) (H&E, 20X).

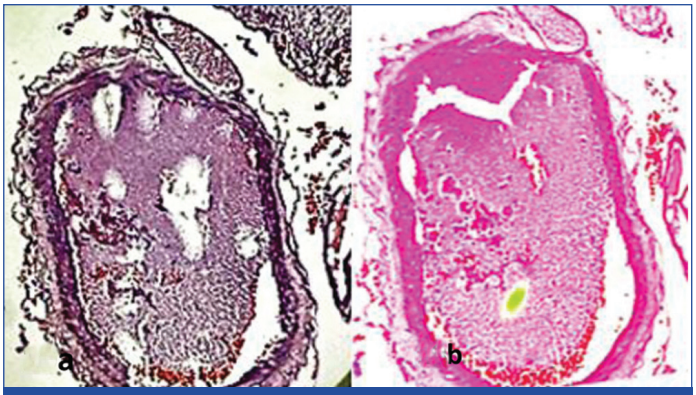
Erythrocytes: Erythrocytes were seen as bright red round to oval structures. In comparison to the surrounding tissue, it displayed excellent contrast. In fact, it appeared better in many cases compared to eosin section [Table/Fig-9].



[Table/Fig-9]: a) Photographs showing artery with RBCs (H&K, 20X); b) (H&E, 20X).

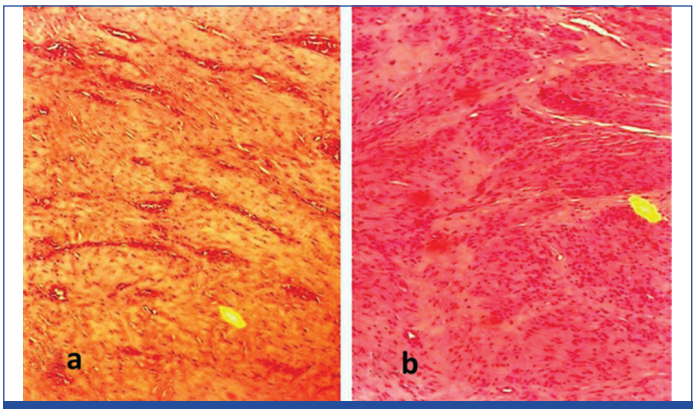
Elastic fibers: Elastic Fibers in the artery were highlighted with dull pink fibers in connective tissue [Table/Fig-10].

Nerve tissue: Nerve tissue showed wavy nucleus with light pink colour.



[Table/Fig-10]: a) Photographs showing elastic fibres in the wall of the artery (H&K, 20X); b) (H&E, 20X).

Smooth muscle tissue: Smooth muscle tissue of Leiomyoma showed bundles and fascicles of dark pinkish red spindle-shaped cells with moderate cytoplasm [Table/Fig-11].



[Table/Fig-11]: a) Photographs showing smooth muscle bundles of leiomyoma (H&K, 20X); b) (H&E, 20X).

DISCUSSION

Stains are a crucial component of laboratory procedure. Synthetic dyes are utilised for the majority of the stain in histology. Although synthetic colours are frequently effective, they can be harmful and cause allergy reactions to some [9]. In India, *kumkum* is a powder with a red colour that is utilised for spiritual and religious purposes. Traditionally, it is made from Crocus sativus L. flowers and lightly spiced with turmeric [10].

The inherent absorption characteristics of distinct tissues with the stain are because of the ionic bond which exists between the dye and the tissue component. Factors playing an important role in this process are the pH of the solution, concentration of the dye and its time of action [11]. In this study, the pH was maintained similar to that of eosin and time was standardised by trial and error method. The *Kumkum* solution was prepared using the maceration technique. Abraham M et al., discovered that when using natural dyes, sections stained using the maceration method yielded better and statistically significant results than sections stained using the soxhlet method, where the vapor of the natural extract is collected [12].

In histopathology, *kumkum* has the double property to stain both nucleus and cytoplasm for a variety of soft and hard tissues. In a study by Lavanya A et al., *Kumkum* staining was used to differentiate various components of oral tissue from bones to soft tissue. The author also highlighted that the incremetal lines displayed superior contrast in *kumkum* staining and as a result they might be used in forensic science. This *kumkum* stain can be used to differentiate between mature and immature bone and can be used to identify bone diseases such fibro-osseous lesions and neoplasms and to evaluate the stages of bone remodelling [6]. Navy N et al., compared the staining characteristics of turmeric and Kumkum sections with those of conventional H&E in 57 cervical tissues and concluded that in H&K stained slides, the cytoplasmic and nuclear features

were better appreciated than H&T and p-value was statistically significant [7].

In our study, connective tissue components such red blood cells, muscle fibres and collagen showed higher contrast in soft tissues slides stained with Kumkum similar to a Nigerian study. This finding has implications for collagen and muscular illnesses [13].

It can also be used in the Pap staining. Cytoplasm showed green to yellow hue of varying intensity depending on the degree of maturation of the cell. Nucleus which can be measured by animage software, appeared greenish-black coloured, but some showed yellowish tinge. Superficial cells showed light pink [14]. Thus, it can be used in routine pap staining also in rapid staining like Diff Quick staining method [15].

Gupta A et al., also yielded a positive outcome of using *kumkum*. The staining procedure produced reddish-pink stained cells highlighted against the white background [16].

The commercially available *Kumkum* powder composition varies with each brand. But in general, it has components like turmeric powder, chalk powder, calcium salts and saffron. Kumkum which is prepared from the saffron flowers of Crocus sativus L. contains 150 volatile compounds and many non volatile compounds. The characteristic components of saffron which includes crocin (responsible for colour), picrocin (responsible for bitter taste) and safranal (responsible for odour and aroma). Saffron has been used as a histological colourant to stain connective tissue [17]. Curcumin has tannins and flavonoids that imparts a brightened tinge [18]. Artifacts due to stain precipitates can be avoided by filtration each time before staining. Some authors have suggested to include microwave heat technique to increase the general staining crispness [19]. More studies must be done to establish its utility in staining malignant cells.

The cost of 25 grams of eosin stain is around 300 in Indian national rupees whereas that of 25 grams of *kumkum* powder is around 75 in Indian national rupees. Therefore, *Kumkum* stain appears to be a cost-effective alternate to eosin stain.

Limitation(s)

One of the limitations is that the durability of the stained *Kumkum* slides was not studied. The slides reviewed after three months showed preserved cell characteristics. Other limitation is only normal tissue architectures are compared, benign and malignant neoplasms are not included.

CONCLUSION(S)

Kumkum solution appears to be an efficient counterstain in place of eosin in high lightening the normal structures in histopathological sections. It proves to be a cost-effective surrogate. It stained the RBCs in the arteries and cytoplasm of the cells in glandular and squamous epithelium better than or equally good as eosin. However, its utility in demonstrating benign and malignant neoplasms in histopathology and durability of the stained slides have to be studied.

REFERENCES

[1] Titford M. Progress in the development of microscopical techniques for diagnosticpathology. J Histotechnol. 2009;32:919.
[2] Alturkistani HA, Tashkandi FM, Mohammedsaleh ZM. Histological stains: A literature review and case study. Glob J Health Sci. 2016;8:72-79.
[3] Fischer AH, Jacobson KA, Rose J, Zeller R. Hematoxylin and eosin staining of tissue and cell sections. CSH Protoc. 2008;2008:pdb.prot4986.
[4] Akinloye AJ, Illoh HC, Olagoke AO. Screening of some indigenous herbal dyes for use in plant histological staining. J For Res. 2010;21:81-84.
[5] Bhuyan R, Saikia CN. Isolation of colour components from native dye-bearing plants in Northeastern India. Bioresour Technol. 2005;96:363-72.
[6] Lavanya A, Sowmya SV, Rao RS, Augustine D, Haragannavar VC. Natural stain (*Kumkum*) formulated by the extract of *Curcuma aromatica* and slaked lime in histostaining of oral tissues: An observational study. J Oral Maxillofac Pathol. 2021;25(1):88-96. Doi: 10.4103/jomfp.JOMFP_90_20. Epub 2021 May 14. PMID: 34349417; PMCID: PMC8272479.
[7] Navya N, Kumarguru BN, Ramaswamy AS, Swethadri GK, Janakiraman P. Utility of Kumkum as a counterstain in histopathological evaluation of cervix: An unexplored archaic behooveful colourant. JCDR. 2021;15(4):EC01-EC06.
[8] Thajudeen A, Srinivasan S, Govindarajan G, Shanmugam A. A comparative study of efficacy of coconut oil, lemon water and dishwashing liquid as surrogates to xylene. Environ Anal Health Toxicol. 2022;37(3):e2022026-0.
[9] Sudhakaran A, Hallikeri K, Babu B. Natural stains *Zingiberofficinale Roscoe* (ginger) and *Curcuma longa* L.(turmeric)-A substitute to eosin. AYU. 2018;39(4):220-25.
[10] Suneetha V, Praveen Kumar G. Kumkuma the social and religious marking of India is used as a stain to improve the contrast of pectinolytic actinomyces VIT S2. Research J Pharm and Tech. 2017;10(5):1297-300.
[11] Kurien BT, Dorri Y, Scofield RH. Spicy SDS-PAGE gels: Curcumin/Turmeric as an environment-friendly protein stain. Methods Mol Biol. 2012;869:567-78.
[12] Abraham M, Nambiar S, Charagannavar V, Augustine D, Sowmya SV, Babu A, et al. Comparison of staining efficacy between turmeric and eosin: A histological study. J Clin Diagn Res. 2017;11(11):05-08.
[13] Avwioro OG, Onwuka SK, Moody JO, Agbedahunsi JM, Oduola T, Ekpo OE, et al. Curcuma longa extract as a histological dye for collagen fibres and red blood cells. J Anat. 2007;210(5):600-03.
[14] Vijayashree R, Rao K. A semi-automated morphometric assessment of nuclei in pap smears using imagej. J Evol Med Dent. 2015;4(53):63-70. https://doi.org/10.14260/jemds/2015/784.
[15] Kannan A, Moorthy PE, Raghavan V, Venkatesan Y. Modified ultrafast Papanicolaou stain: Benefits and challenges over conventional Papanicolaou stain in cytological diagnosis. Ann Trop Med Public Health. 2020;23:231-506.
[16] Gupta A, Sengupta S, Nathan A, Agarwal A, Devadas M, Madhumathi G, et al. Preparation of a microbial stain from the natural product kumkum for pharmaceutical applications. Int J Drug Dev Res. 2014;6(4):218-21.
[17] Srivastava R, Ahmed H, Dixit RK, Dharamveer S, Saraf SA. Crocus Sativus L: A comprehensive review. Pharmacogn Rev. 2010;4(8):200-08.
[18] Suryawanshi H, Naik R, Kumar P, Gupta R. *Curcuma longa* extract- Haldi: A safe, eco-friendly natural cytoplasmic stain. J Oral Maxillofac Pathol. 2017;21(3):340-44. Doi: 10.4103/jomfp.JOMFP_62_17. PMID: 29391705; PMCID: PMC5763853.
[19] Priya AH, Venkatanarasu VB, Chellaswamy S, Jeyaraj M, Francis SF, Rajasekaran S. Evaluation of efficacy of microwave staining over conventional staining in replicating tissue architecture: A prospective study. J Pharm Bioall Sci. 2020;12:S283-88.

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