ORIGINAL ARTICLE

Hibiscus: A different hue in histopathology
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Abstract
Background: Nature is vibrant and has an abundance of colors. The use of natural hues in histopathology could offer an economical and feasible alternative to the routinely used stains in special circumstances.

Aim: To evaluate the efficacy of the extract of Hibiscus as a counterstain to hematoxylin.

Methodology: Hibiscus calyces were sun dried and powdered. Alcoholic and water extracts were obtained by refluxing the powder with 95% ethanol and distilled water, respectively, for 3 h, and then cooled and filtered. Sections from 12 formalin-fixed paraffin-embedded tissue blocks, including both oral mucosal and skin tissues, were used. Nuclear staining was done with hematoxylin, following which the sections were stained with both alcoholic and aqueous Hibiscus extract for 10 min at room temperature using a dropper. All the slides were coded and were reviewed by three blinded oral pathologists for staining efficiency and intensity. NPar test, Kruskal-Wallis test, and Kappa statistics were done to assess the staining efficiency, intensity, and interobserver reliability for the selected parameters.

Results: Statistically significant difference was not seen between Hibiscus extract and the controls, except in relation to staining of the basement membrane.

Conclusion: Easily available and economical Hibiscus extract is an attractive alternative to eosin. Further studies involving the addition of mordants to the extract and its use as a special stain has to be explored.

Keywords: Counterstain, hematoxylin, Hibiscus, Hibiscus rosa-sinensis

Introduction

There can be no two thoughts about the pre-eminent status of hematoxylin and eosin (H and E) in histopathologic staining. H and E staining has facilitated pathologists over the years to get familiar with the cellular “acquaintances.” It is only under special circumstances that other stains are commissioned and hence justifiably earning the sobriquet - “Special stains.” Any stain trying to emulate H and E has to invariably justify the attempt through its performance and/or practicality.

Hibiscus is a genus of plants composed over hundred species of Hibiscus known for their colorful and showy flowers. Hibiscus calyces are rich in anthocyanin, ascorbic acid, and Hibiscus acid.\textsuperscript{1,2} The bright color of the Hibiscus calyces is by virtue of the presence of anthocyanin.\textsuperscript{1,2} Flower extracts of some species have been used extensively as natural dyes in the textile industry where they are known for their vibrant and long lasting color.\textsuperscript{1,2} The calyces have also been used as food colorants in jams, juices, pharmaceutical syrups, and as an emulsifier in carbonated drinks.\textsuperscript{1,2} Studies exist on the application of extract of Hibiscus sabdariffa, as a histopathological stain and staining of fungi and parasites.\textsuperscript{3,4} However, Hibiscus rosa-sinensis, another species of Hibiscus is found in abundance in the tropics, including India.\textsuperscript{2} The plant species is found aplenty in and around Coorg district. Limited studies are available on the staining properties of this plant in routine histopathology. The present study aimed at evaluating the efficacy of H. rosa-sinensis extract as a cytoplasmic stain as a viable alternative for eosin.

Methodology

Preparation of Hibiscus extract

Hibiscus calyces were sun dried and powdered. From this powdered form, alcoholic and water extracts were obtained. Alcohol extract was obtained by refluxing 40 g of powder in 500 ml of 95% ethanol for 3 h, and then cooled and filtered. The original pH of the alcoholic extract was 5.7. The aqueous extract was obtained by
refluxing 50 g of powder in 600 ml of distilled water for 3 h, and then cooled and filtered. pH of the aqueous extract was 5.5.

**Sectioning and staining methodology**
Sections from 12 formalin-fixed paraffin-embedded tissue blocks, including both oral mucosal and skin tissues, were used. 5 µ thick sections were cut using semi-automatic soft tissue microtome. Sections were de-waxed, taken through different grades of alcohol. Nuclear staining was done with hematoxylin, differentiated with 1% acid alcohol, following which the sections were stained with both alcoholic and aqueous Hibiscus extract for 10 min at room temperature using a dropper. Sections stained with H and E acted as controls.

All the slides were coded and were reviewed by three blinded oral pathologists for staining efficiency and intensity. A preliminary evaluation was undertaken to compare staining efficiency between alcoholic extract and aqueous extract at different pH values: 3.7, 5.7, and alkaline pH of 12.7. The staining efficiency was also assessed using microwave and incubator staining methods. Staining intensity was assessed semi-quantitatively by assigning grades as + for satisfactory, ++ for intermediate, and +++ for good staining intensity. The parameters assessed were as listed in Table 1.

**Statistical analysis**
NPar test, Kruskal-Wallis test, and Kappa statistics were done. The P value was set at 0.05. About 12 sections each from the control group and Hibiscus (alcohol extract, pH - 5.7) were selected and were assessed for staining efficiency, intensity, and interobserver reliability.

**Results**
On the evaluation of the stained sections, it was found that the ethanolic extract of Hibiscus performed better than the aqueous extract. The alcoholic extract at pH of 5.7 showed better contrast to hematoxylin and better staining intensity [Figures 1-3]. The statistical tests showed no significant difference between H and E and Hibiscus staining with respect to all but one of the selected parameters (Tables 2 and 3). Staining of the basement membrane showed a significant difference (P = 0.011) between the control and Hibiscus staining [Table 2]. Interobserver reliability for the control group and Hibiscus group was good with a Kappa score of 0.94.

**Discussion**
Hibiscus has long been used as a natural dye in food and beverages, in the cosmetic industry as a hair dye, and in dyeing fabrics and textiles. It renders vibrant colors owing to the presence of anthocyanins and is well-known for the color stability and fastness. Researchers have applied this property of Hibiscus dyes in histopathology and have used it for staining fungal species, parasites, neural tissue, and sperm cells and found the staining properties to be satisfactory. The present study attempted to utilize the extract of locally available H. rosa-sinensis, a histopathological stain with hematoxylin as a counterstain.
The alcohol extract of *Hibiscus* was found to have a better contrast to hematoxylin, better staining intensity in comparison with the aqueous extract. Alcoholic extracts have been used with considerable success to stain fungi. In this study, we used both the alcoholic and aqueous extracts at different pH. On preliminary evaluation, ethanolic extracts at pH 5.7 gave optimal staining both in the epithelium and the connective tissue. pH modification of dyes is known to affect their staining quality. Egbujo *et al.*, in their study, found modification of aqueous extract of *H. sabdariffa* to alkaline pH gave the poorest results.

The ethanolic extract (pH 5.7) stained sections were further compared with H and E stained sections. The staining contrast and intensity were comparable to H and E, and there was general agreement in the assessment of the three oral pathologists. Microwave stained sections in our study offered better intensity with slight compromise in the cellular contrast. Domestic microwave, as used in our study, has been found to provide staining quality equal to or superior than the standard methods of the many routinely used histopathology stains. The comparatively longer staining time of *Hibiscus* extract can also be easily circumvented by the use of microwave staining.

The results seem to suggest the parallel staining quality of *Hibiscus* extract with regards to eosin. The advantages of using *Hibiscus* would be its ease of availability, extraction, and modification. Literature also suggests the versatility of *Hibiscus* staining. The extract also has been used as a special stain for parasites, fungi, and neural tissues. *H. sabdariffa* also has been mordanted with iron alum or potassium alum and used with eosin as a counterstain. However, the main limitation of using *Hibiscus* would be the pathologists’ preference toward eosin due to habituation.

**Conclusion**

In conclusion, easily available and economical *Hibiscus* extract offers an attractive alternative to eosin. However, long-term retention of *Hibiscus* stain and its use as a special stain have to be further evaluated. Modification of the stain with the use of mordants and its compatibility with different counterstains has to be explored.

**References**