

# **Iron-Roselle: A Progressive Nuclear Stain for Connective Tissue of Skin**

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and Olutunde OA<sup>1</sup>**

## **Abstract**

**Objective:** this is a comparative experimental study aimed at exploring the efficiency of Hibiscus sabdariffa (roselle) as natural staining dye for the histological demonstration of skin morphology and its connective tissue as compared to the routinely used haematoxylin and eosin.

**Methods:** 10% neutral buffered formalin fixed, paraffin embedded tissue blocks of skin were retrieved from the tissue block archive of the Pathology Department, Unilorin Teaching Hospital. Two blocks were randomly selected. Twelve serial sections were cut from each and six labeled A & B each for each block. Sections labeled A were stained with 5% concentration of H. sabdariffa solution and B stained with H&E as parallel control.

**Result:** Photomicrographs from each group demonstrated the histo-morphology and connective tissue component of skin in comparable and almost indistinguishable manner.

**Conclusion:** This study established the capability of Hibiscus-iron/eosin to replace H&E in the morphological and connective tissue demonstration of skin. Hibiscus sabdariffa is readily available locally, safe, easily prepared and resists fading.

**Keywords:** Hibiscus Stain, iron-Roselle, Histological Staining, natural dye, Skin

## **1 Introduction**

Development of new histological staining method remains justified especially if the

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new stain is harmless, easy to use, cheap, commercially favorable, and gives the desired practical results [1].

Hematoxylin and Eosin (H&E) stains play a critical role in tissue-based diagnosis [2]. The two, are the commonest stains being used in histopathology laboratories all over the globe which colour the nuclei a dark blue or purple, and the cytoplasm and connective tissue a shades of pink [3]. By colouring tissue structures (cytoplasm, nucleus, organelles, and extra-cellular components); these stains allow the sections to be viewed in details under the microscope, tissue morphology and/or any present abnormalities can then be easily detected [4]. Even when advanced staining methods are used, the H&E stain still forms a critical part of the diagnostic picture as it displays the underlying tissue morphology which allows correct interpretation to be made [5]. Most histological stains in current use are of synthetic origin; however, natural dyes are still promising to be cheaper potential sources [6].

*Hibiscus sabdariffa* belongs to the vascular flowering plants, known as Roselle or Red Sorrel in English and Karkade in Arabic [7]. *Hibiscus Sabdariffa* is a plant cultivated in many countries in the world; Sudan and Nigeria are among these countries. In Sudan, it is called Karkade; the extract is usually used as a drink, hot tea-like in winter and cold in summer. In Nigeria, it is called zobo and used as refreshment [8,9].

The skin is the largest organ in the body, both in weight and surface area, and shows marked variation in structure at different sites in the body surface {10}

The extraction and application of colouring matters from *Hibiscus sabdariffa* (Roselle) will be a great contribution to the exploitation of natural dyes in the demonstration of skin histomorphology and dermatological studies. This research want to contribute to the use of natural dyes from *H. sabdariffa* (Roselle) as a substitute to haematoxylin in H&E in the staining of human and animal skin tissues especially in the field of dermatology.

## 2 Methodology

Dry leaves of *Hibiscus sabdariffa* were purchased at a local market in Ilorin, Kwara State, Nigeria and were identified by a Botanist in the Botany department of Obafemi Awolowo University, Ile-Ife, Oyo State, Nigeria. They were processed using the technique of Benard (9). 10% neutral buffered formalin fixed paraffin processed skin tissues were sectioned at 4 microns. Slides of serial sections produced were tagged A and B. A: Haematoxylin and Eosin B: *Hibiscus* and Eosin.

The slides were further coded and reviewed by three blinded histopathologists for staining quality and intensity. This was done semi-quantitatively by assigning grades + for satisfactory, ++ for good and +++ for very good staining quality and intensity. The parameters assessed include nucleus /cytoplasm contrast, tissue contrast (cells versus background) and vasculature.

## **1. PREPARATION OF STAIN EXTRACTS**

### **1.1. HIBISCUS EXTRACT SOLUTION**

The dry calyces of *Hibiscus sabdariffa* were ground using a Binatone blender to a fairly powdery form. To 10g of the ground red calyces of *H. sabdariffa* in a conical flask, 200ml of distilled water was added and brought to boil to give a brilliant red coloured extract which was immediately allowed to cool and filtered to give a clear *H. sabdariffa* extract. The staining formular was compounded as follows:

*H. sabdariffa* extract: 100ml

NaCl 5.0g

Glacial acetic acid 3.0ml

### **1.2. 1% Alcoholic Eosin**

1g of eosin yellow was weighed on a digital Ohaus balance, dissolved in 95% ethanol and made up to 100ml.

## **2. STAINING PROCEDURES**

### **2.1. H&E Staining Procedure**

1. Dewax in xylene and hydrate through 100%, 90%, 70%, 50% alcohol to water
2. Stain section in Harris haematoxylin for 15 minutes
3. Rinse in water
4. Differentiate in 1% acid alcohol
5. Blue in running tap water for 10 minutes
6. Counter-stain in 1% alcoholic eosin for 60 seconds
7. Dehydrate in ascending grades of alcohol
8. Clear in xylene
9. Mount in DPX

### **2.2. Hibiscus/Eosin Staining Procedure**

1. Dewax in xylene and hydrate through 100%, 90%, 70%, 50% alcohol to water
2. Stain in Hibiscus extract solution for 5 minutes
3. Wash in running tap water for 2 minutes
4. Counter-stain in 1% alcoholic eosin for 30 secs
5. Dehydrate in ascending grades of alcohol
6. Clear in xylene
7. Mount in DPX

### 3 Main Results

The Hibiscus-eosin staining shows blue-black nuclear staining while the H&E staining shows purplish-blue nuclear staining. Cytoplasm component stains red with Hibiscus-eosin as for H&E. Histomorphology of skin is clearly demonstrated with the new technique and distinct presentation of intercellular membranes comparable with H&E (Fig. I&II).

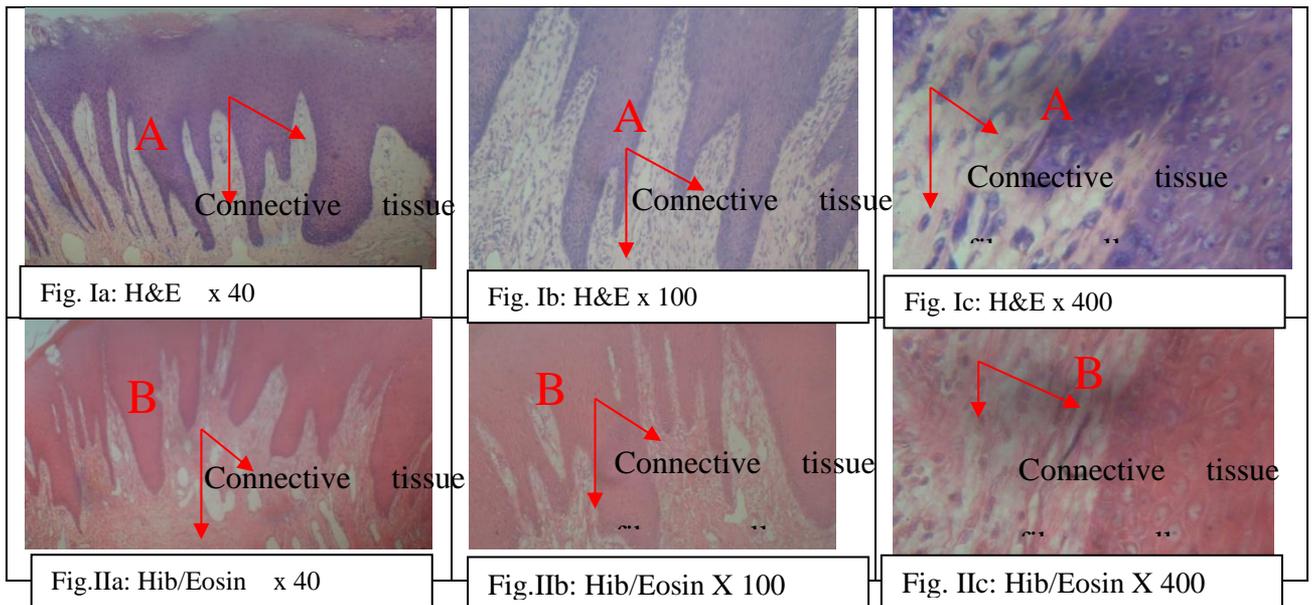


Figure: Photomicrographs of the sections of group A and B are shown above as designated

The histomorphological features of the epidermis and dermis as well as the connective tissue were distinctly preserved.

### 4 Conclusion

Few works exist on the application of iron-Roselle as a progressive nuclear stain in histopathology. Some earlier workers tried to use the watery extract of Hibiscus sabdariffa (Roselle) as cytoplasmic stain to substitute for eosin in H&E staining technique {11, 12, 13} (Hashim, 2006; Egbujo et al., 2008, Ibnouf et al., 2014, ).

Another group of early researchers canvassed the use of H. sabdariffa extract as

nuclear stain for histological demonstration of tissues {9, 12,14, 15, 16} (Benard, 2008; Egbujo et al., 2008; Benard, 2015; Benard et al., 2015; Muhammed et al., 2016; Benard et al., 2016). Benard and colleagues (2016) {16} recommended its use as a progressive nuclear stain while Egbujo et al., (2008) {12} reported its use as regressive nuclear stain.

Quality of staining in histology depends on the microscopic appearance of cell membrane, nuclear membranes, cytoplasm transparency, and extracellular matrix {17} ( Raheem et al., 2015)

H. sabdariffa renders vibrant colour owing to the presence of anthocyanins and is well known for the colour stability and fastness {18} (Sridhara et al., 2016). Researchers have applied this property of Hibiscus dyes in histopathology and have used it for staining fungal species, parasites, neural tissue, and sperm cells. They found the staining properties to be satisfactory {18}(Sridhara et al., 2016). Hibiscus extract-iron solution has also been found useful in the staining of lymphnode, appendix, liver, kidney, brain and hippocampus {15, 16} (Benard et al., 2016 and Muhammed et al., 2016).

The present study attempted to use the extract of locally available H. sabdariffa extract, mordanted with iron chloride solution counterstained with eosin as a histological stain to demonstrate connective tissue of skin.

Independent assessment by three histopathology experts scored connective tissue staining by the iron-Roselle technique as good (++) . Nucleus –cytoplasm contrast was also rated to be satisfactory (+) and good (++) . Similar assessment was apportioned for red blood cells. An unsuspecting observer does not usually question whether the section under his microscope is stained with H&E or Iron-Roselle/Eosin.

Details of the mechanism of staining need to be further investigated but it is suggested that the ferric ions which make the dye more nucleus – specific combine with the anthocyanin pigment (flavonoids) of Roselle which confers a net positive charge on the dye (Benard, 2008). The cationic dye metal complex binds with the anionic nuclear chromatin to give blue-black colour. The counterstain eosin, which is an anionic dye combines electrostatically with the cytoplasm and other tissues. Optimal staining of 5-20 minutes was earlier recommended {9} (Benard, 2008). However, this work has achieved 5 minutes optimal staining thus confirming its applicability as a progressive stain. The staining solution is best stored at 4<sup>0</sup>C where it remains viable for three months. The main limitation of using Iron-Roselle (Hibiscus) progressive stain would be the pathologists' preference for haematoxylin due to habituation.

Conclusively, Iron-Roselle is a suitable progressive nuclear stain that could substitute for haematoxylin in the demonstration of connective tissue of skin. It could therefore be a useful technique in diagnostic dermatology.

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