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Regressive Hibiscus sabdariffa Extract Nuclear Stain Using Formalin Fixed Paraffin Embedded Skin, Lymph node and Appendix

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Abstract

A simple boiling water extract of Hibiscus sabdariffa was compounded with 10% ferric chloride solution, sodium chloride and glacial acetic acid and stored at 4°C. Formalin fixed paraffin embedded blocks of skin, lymph node and appendix were randomly retrieved from the tissue block archive of the Pathology Department, University of Ilorin Teaching Hospital, labeled and sectioned at three microns. Sections were stained with the Hibiscus-iron solution in line with routine protocol for haematoxylin and eosin. Parallel control for each organ was done using Ehrlich's haematoxylin. Microscopic slide review by histopathology experts revealed comparable staining with standard haematoxylin and eosin thus establishing the suitability of Hibiscus extract solution as a regressive stain in histopathology laboratory especially in the demonstration of skin, lymph node and appendix.

Keywords: *Hibiscus sabdariffa*, *histology stain*, *natural dye*, *lymph node*, *appendix*, *skin*

Introduction

Staining techniques are necessary as contrast media if biological and histopathological tissues are to be demonstrated (Avwioro, 2011). Haematoxylin is the most commonly used stain in histopathology laboratory globally and it is obtained from the logwood of haematoxylon campechianum, cultivated in Mexico (Rahul *et al.*, 2019). On its own, haematoxylin cannot stain tissues except when oxidized to haematein and combined with mordants such as aluminium, iron, tungsten and molybdenum in order to

increase its affinity to tissues (Avwioro, 2011).

Haematoxylin techniques has affinity for nuclei and useful for the demonstration of connective tissue fibres, myelin sheath, carbohydrates, cells in exfoliative cytology, etc (Avwioro, 2011). It is also applicable in immunohistochemistry as a contrasting agent (Grosset *et al.*, 2019). The dye is durable, amenable to differentiation and has permanent staining capability (Rahul *et al.*, 2019).

Eosin is the most commonly used counterstain for haematoxylin stains. This xanthene dye is commonly available as Eosin Y or B but Eosin Y is popularly used as 0.5-1% aqueous or alcoholic solution (Suvarma *et al.*, 2013).

The hazardous nature of synthetic haematoxylin, its non-eco-friendliness, periodic scarcity in resource limited countries coupled with the perpetual undulating downturn in the economies of third world countries made the search for locally available, cheap, eco-friendly and easily prepared alternatives attractive (Agbede *et al.*, 2017; Benard *et al.*, 2015).

Plants being rich in natural dyes extracted from their leaves, bark, fruit, seeds, flowers, and roots are good candidates for exploration (Rahu *et al.*, 2019). One of such plant is Hibiscus sabdariffa which is cultivated in Asia and most countries in the tropics especially Sub-Saharan region of Africa (Benard, 2008). In Nigeria, it is widely distributed in the South West, North Central, and North West and North East regions (Daudu *et al.*, 2015).

Several names are associated with the plant globally. In Iran, it is called Kujurat flower, "Oseil de rouge in France, Karkade in Egypt (Benard *et al.* 2015) and Isapa among the Yoruba speaking South Western States of Nigeria (Adegunloye, 1996).

Various efforts have been made globally to find useful ways of applying *H. sabdariffa* in the demonstration of tissues. A staining combination of picric acid and *H. sabdariffa* extract was used for degenerated muscle fibre and recommended for muscle pathology (Gowali, 1996). A research work on testicular tissues of rats reported enhanced nuclear and cytoplasmic staining using extract of *H. sabdariffa* mordanted with potassium alum or iron mordant combined with eosin counter stain (Egbujo *et al.*, 2008). A dark violet nuclear staining was reported in a study which used aqueous extract of the dye on brain and skin tissues (Agbede *et al.*, 2017; Benard *et al.*, 2015). Another study reported a dark violet nuclear staining with rat hippocampus using the extract of *H. sabdariffa* (Ola *et al.*, 2016).

Previous works on *H. sabdariffa* iron extract solution as nuclear stain in histopathological demonstration of tissues emphasized on progressive method of staining. This current work reports its use as a regressive stain on formalin fixed, paraffin embedded skin, lymph node and appendix.

Methodology

Preparation of *H. sabdariffa* iron solution

Ten grams (10g) of ground dry calyx of *H. sabdariffa* purchased at a local market in Ilorin Kwara State, Nigeria was poured into a conical flask and dissolved in 200ml of distilled water and brought to boil on a Bunsen burner for five minutes. The resultant red solution was allowed to cool and filtered into a brown bottle. One hundred milliliters (100ml) of the *H. sabdariffa* extract was mixed with 5g of sodium chloride, 1.2. ml of 10% anhydrous ferric chloride solution and 3ml of acetic acid. Storage was done at 4°C.

Sectioning

Formalin fixed, paraffin embedded tissue blocks were retrieved from the archive of the Pathology

Department, University of Ilorin Teaching Hospital and sectioned at three microns using a Leica Microtome. Lymph node sections were labeled A, Skin sections B and Appendix C.

Sections were stained with formulated Hibiscus technique and H&E as parallel control.

Staining

Sections were dewaxed in xylene and taken through descending grades of alcohol and rinsed in water. Staining in *H. sabdariffa* solution was done for 15 minutes, differentiated in 1% acid alcohol, rinsed and blued in running tap water for 10 minutes, rinsed in water and counter stained in 1% alcoholic eosin for 30 seconds. Stained sections were dehydrated in ascending grades of alcohol, cleared in xylene and mounted in DPX. Parallel sections were stained with standard routine H&E.

Verification and Validation

Stained slides were reviewed and validated by three histopathology experts.

Results

Hibiscus sabdariffa regressive technique stained nuclei violet-blue and cytoplasm, pink. Red blood cells stained red and collagen, pink. This is comparable to the staining by standard Harris haematoxylin.

Discussion

Regressive staining techniques are meant to over stain tissue components so that at a later time, differentiation using weak mineral acids such as 1% acid alcohol could be introduced to break the dye-tissue bond in weakly stained areas and that at the end of the day, acceptable nuclear staining is achieved (Avwioro, 2011).

Aluminium haematoxylin formulations such as Harris haematoxylin have been commonly applied in histopathology laboratories globally as a regressive and progressive stain (Avwioro, 2011). It is also consistent when combined with eosin. It was for these reasons it was adopted as parallel control for this research.

Previous works on *H. sabdariffa* laid emphasis on its use as a progressive stain. More than a decade ago, Hibiscus –iron solution was used as



progressive method to demonstrate lymph node and appendix (Benard, 2008). In another study by Ola *et al.* (2016), H. sabdariffa iron solution was used as a progressive stain in combination with extract of Sorghum bicolor and recommended as a morphological stain in neurological studies. Further application of H. sabdariffa iron solution as a progressive stain was demonstrated in combination with van Gieson counter stain as a neuro-histological stain (Benard *et al.*, 2016). Recently, another group of researchers, (Agbede *et al.*, 2017), used it as a progressive Hibiscus staining technique to demonstrate skin morphology.

Contrariwise, a study by some group of researchers (Egbujo *et al.*, 2008) had recommended the use of H. sabdariffa-alum-mordanted techniques as stain for the demonstration of testes but a clear-cut formulation and staining procedures to demonstrate reproducibility was not made available. Another study used different formulations of hibiscus extract to stain skin, testes and to assess bar bodies (Omorodion, 2017). In their report, simple hibiscus extract counter stained with eosin was adjudged the most comparable to H&E especially in staining testes. Lack of a clear-cut procedure of staining makes it difficult to understand whether the authors used the local extract as progressive or regressive stain.

This work explored the applicability of H. sabdariffa iron solution as a regressive stain treated the same way standard regressive Ehrlich's haematoxylin and eosin was treated. The good result obtained despite using 1% acid alcohol instead of ferric salt to differentiate is quite encouraging. The principle of staining depends on the hibiscus-dye-lake complex formed by the hibiscus dye and 10% ferric chloride solution. This basic complex has affinity for acidic nuclear component of the tissue and stains it violet-blue. Preliminary qualitative phytochemical study in this work showed that hibiscus extract contains flavonoids, tannins, phenols, protein, and terpenoid. The nuclear component of the tissues; lymph node, appendix and skin appeared violet-blue showing the morphological characteristics of the

organs convincingly. (See Fig. 1, 2 and 3).

The lymph node stained with the Hibiscus solution showed reactive lymphocyte cells, cortical spaces and connective tissue collagen (Fig. 1); the appendix showed sub-mucosa aggregated lymphocytes, adipose cells and loose connective tissue meshwork in a background of red blood cells (Fig. 2) while skin showed the dermis, epidermis and dense collagen connective tissues (Fig. 3). The features are so unmistakable that the way it mimics standard H&E stain is never in doubt by observers.

The cytoplasm of organs stained with H. sabdariffa solution also gave good contrast appearing pink comparable to pink for standard haematoxylin. Impressive grading of the slides by histopathology experts (See table 1) shows the acceptability and versatility of the Hibiscus sabdariffa iron solution staining technique. This showed that it has the potential of being used when imported chemicals for the compounding of Harris haematoxylin is not available.

The local availability, ease of preparation and use, eco-friendliness and less hazardous nature of Hibiscus iron solution staining technique makes it attractive as a substitute to Harris haematoxylin as regressive staining technique in histopathology laboratories especially for skin, lymph node and appendix.

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Table 1: Staining quality of organs by Hibiscus sabdariffa solution compared with standard H&E

Organ/ Technique	Nuclei	Cytoplasm	RBC	Collagen	Adipose cells	Lymphocytes
Lymph node						
H&E	+++	+++	+++	+++	+++	+++
Hib/Eosin	+++	+++	+++	+++	+++	+++
Appendix						
H&E	+++	+++	+++	+++	+++	+++
Hib/Eosin	+++	+++	+++	+++	+++	+++
Skin						
H&E	+++	+++	+++	+++	+++	N/A
Hib/Eosin	+++	+++	+++	+++	+++	N/A

+++ = Very Good N/A = Not applicable

Figures

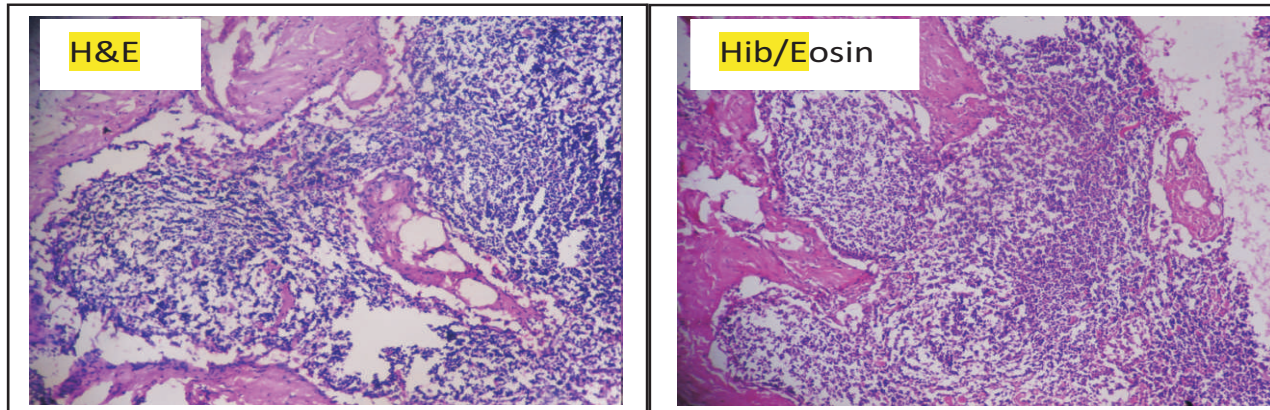


Fig. 1: Lymph node showing H&E and Hibiscus/ eosin staining of lymphocytes, cortical spaces and collagen connective tissues. The comparable staining of Hibiscus solution with standard H&E is outstanding. Mg x 100

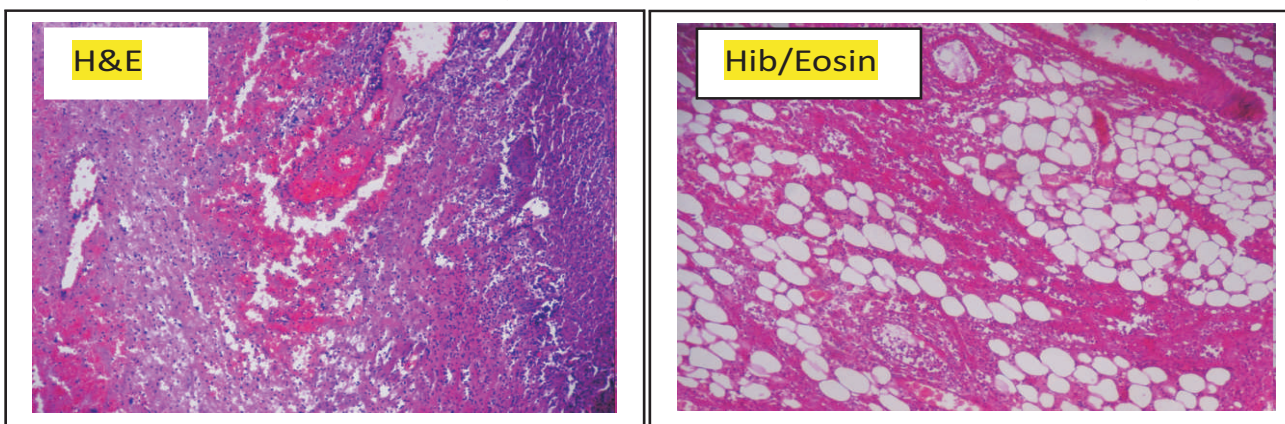


Fig. 2: Appendix showing H&E and Hibiscus/ eosin staining of lymphocytes, adipose cells and sub mucosa collagen connective tissues and red blood cells. Mg x 100

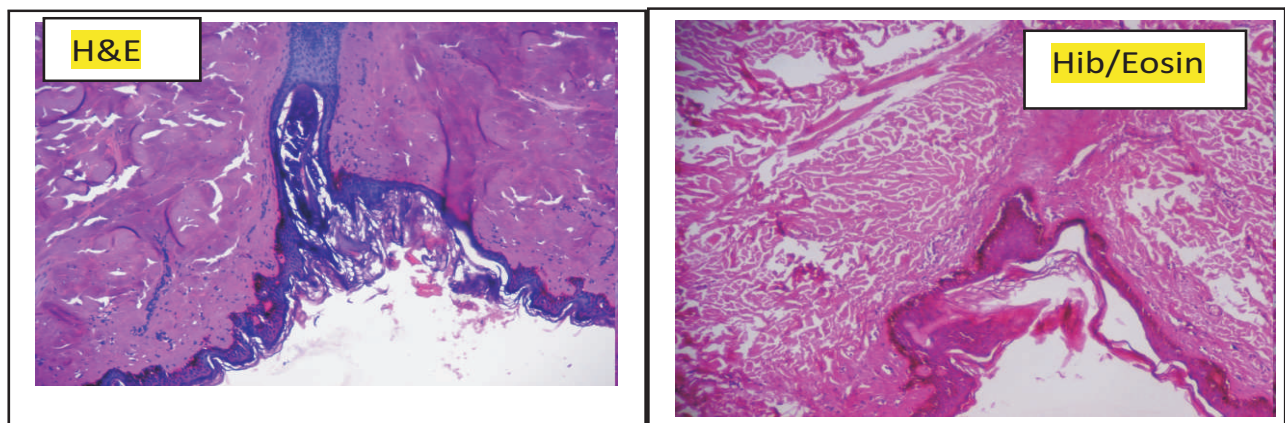


Fig. 2: Skin showing H&E and Hibiscus/ eosin staining of epidermis, keratin, collagen connective tissues and red blood cells. Mg x 100

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