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Hibiscus-Trichrome Stain for Collagen, Muscle and Red Blood Cells in Skin TissueBenard, S.A.^{1*}, Fowotade, A.A.¹, Olutunde, O.A.¹, Afolabi, O.O.²Histopathology Laboratory Unit, Department of Pathology, University of Ilorin Teaching Hospital, Ilorin, Kwara State, Nigeria¹, Department of Pathology, College of Health Sciences, University of Ilorin, Ilorin, Kwara State, Nigeria².

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Abstract

The use of Hibiscus sabdariffa (Roselle) as natural histological stain has gained currency in recent times especially as a nuclear dye alternative to haematoxylin. This comparative experimental study explored the capability of Hibiscus mordanted with Ferric Chloride and counter stained with Ponceau 2R and Light Green as collagen fibres, muscle and red blood cells stain in comparison with standard Masson Trichrome (MT) used as parallel control in skin tissue. Microscopic study reveals satisfactory staining of the connective tissue elements by the new technique as compared with the standard method. The local availability of H. sabdariffa and ease of preparation is a justification for its use as a substitute for haematoxylin formulation in routine Masson Trichrome technique especially in dermato-architectural studies and diagnoses.

Keywords: *Hibiscus, roselle, trichrome, skin, collagen*

Introduction

Staining methods that will simultaneously color various tissue components in a histological section have long appealed to histopathologists and there are a multitude of histological methods that give multiple colors (Michael, 2009). The first reported triple stain was that of Gibbes in 1880 (Bracegirdle, 1986). However, a better-known triple stain was Ehrlich's 'triacid stain' published in 1888 (Clark and Kasten, 1983) which used methylene blue, acid fuchsin, and Orange G, a stain with which led to the discovery of granulation in neutrophils. A trichrome in

today's histopathology laboratory usually means a section with the nuclei stained with hematoxylin, and the connective tissue and cell cytoplasm stained a different color, rather than just a section with three colors (Clark and Kasten, 1983). The most popular stain in present times is the Masson's Trichrome introduced in 1929 (Sheehan and Hrapchak, 1980) which stains nuclei blue-black, cytoplasm red and connective tissue green or blue depending on the particular stain chosen (Clark and Kasten, 1983). The prices of imported reagents for histopathology laboratory use have skyrocketed in recent times especially in developing countries whose resources are limited. As it first occurred in the 70's (Al-Tikriti and Walker, 1978), hematoxylin has shown one of the highest increases and now costs multiple times more than it did globally in 1973. This situation is worse in developing countries particularly in Asia and Sub-Saharan Africa. These events have justified considerable efforts to find an effective local hematoxylin substitute in histopathology techniques for use in histopathology laboratories in resource limited settings.

Early reports showed that Hibiscus extract could substitute for hematoxylin in the demonstration of different tissues and organs (Benard et al., 2016; Muhammed et al., 2016; Benard et al., 2015a; Benard et al., 2015b; Benard et al., 2008; Egbujo et al., 2008). Yetunde and colleagues (2017) also carried out a study of the staining effect of H. sabdariffa in demonstrating the histologic features of lymphoid tissues. In the same vein, another team of researchers (Omorodion et al., 2017) investigated the use of

H. sabdarifaa as a histological stain and in the assessment of bar bodies. Findings in these regards prompted a systematic study to determine the capability of Hibiscus extract being used in trichrome staining technique as a substitute for the routinely used, hematoxylin –dependent, Masson trichrome histochemical stain especially to demonstrate collagen fibers, muscle and red blood cells of human skin tissue. There is paucity of information on the use of hibiscus dye as a trichrome stain.

Materials and Methods

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 and Colleagues (Benard et al., 2016). Ten

percent (10%) neutral buffered formalin fixed, paraffin embedded skin tissues were retrieved from the archive of the Histopathology Laboratory, University of Ilorin Teaching Hospital and sectioned at 4 microns. They were later stained with Hibiscus-Trichrome method. Parallel sections were stained with Masson's Trichrome and H&E as controls.

Preparation of Hibiscus Extract

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 the brilliant red colored extract which was immediately allowed to cool and filtered to give a clear *H. sabdariffa* extract. The staining formula was compounded as follows:

Component	Quantity
NaCl	5.0g
<i>H. sabdariffa</i> extract	100ml
10% ferric chloride solution	1.2ml
Glacial acetic acid	3.0ml

Hibiscus-Trichrome Staining Technique

Sections were dewaxed in xylene and hydrated through 100%, 90%, 70%, 50% alcohol to water and subsequently stained in Hibiscus extract solution for 5 minutes, washed in running tap water for 10 minutes, stained in acidified Ponceau 2R for 5 minutes, rinsed in distilled water, differentiated and mordanted in 1% phosphomolybdic acid for 5 minutes, drained and put in acidified light Green for 5 minutes, differentiated in 1% acetic acid for 1 minute, blotted with filter paper, dehydrated in hot air oven, cleared in xylene and mounted in DPX.

Masson's Trichrome Staining Technique

Sections were dewaxed in xylene and hydrated through 100%, 90%, 70%, 50% alcohol to water and subsequently stained in equal volumes of Wiegert's A and B for 20 minutes, differentiated in 1% acid alcohol, washed in water for 10

minutes, stained in acidified Ponceau 2R for 5 minutes, rinsed in distilled water, differentiated and mordanted in 1% phosphomolybdic acid for 5 minutes, drained and put in acidified light Green for 5 minutes, differentiated in 1% acetic acid for 1 minute, blotted with filter paper, dehydrated in hot air oven, cleared in xylene and mounted in DPX.

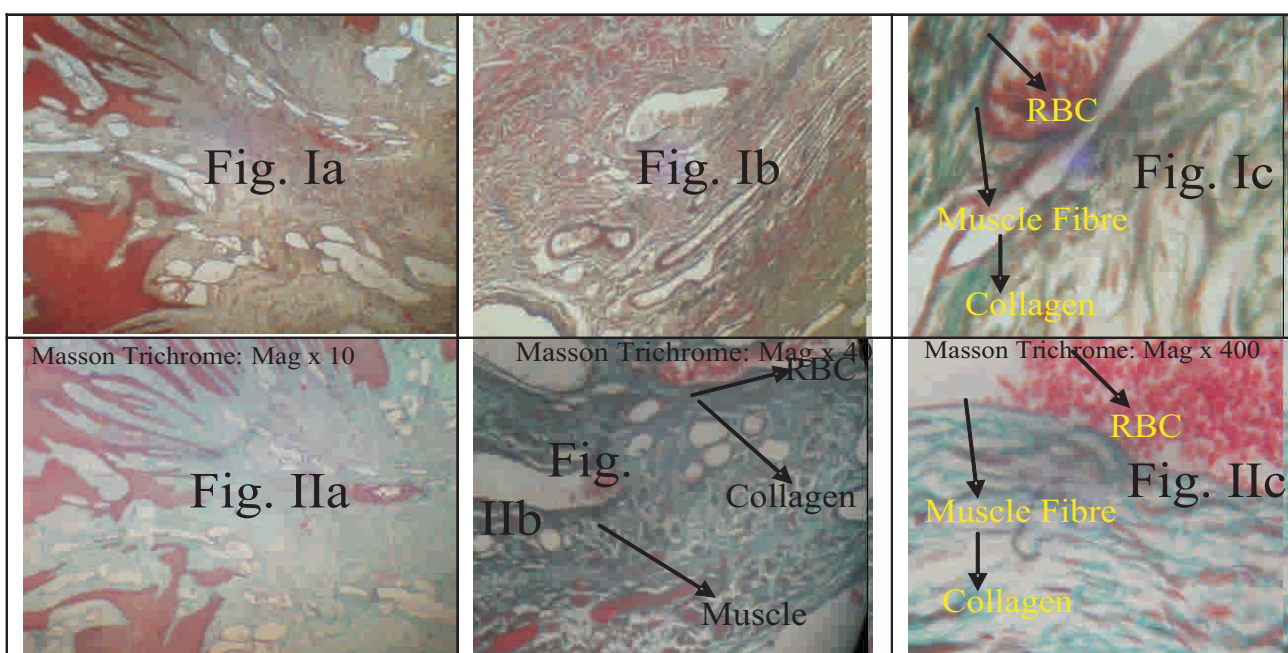
H&E Staining Technique

Sections were dewaxed in xylene and hydrated through 100%, 90%, 70%, 50% alcohol to water and subsequently stained in Harris hematoxylin for 15 minutes, washed in running tap for 2 minutes, differentiated in 1% acid alcohol, blued in running tap water for 10 minutes, counter stained in 1% alcoholic eosin for 30 seconds, dehydrated in hot air oven and mounted in DPX.

Result

Nuclei appear black, collagen green, muscle red and red blood cells, red. This compares

satisfactorily with that of standard Masson's Trichrome.



Hib- Trichrome: Mag x 40 Hib-Trichrome: Mag x 40 Hib-Trichrome: Mag x 40

Figure I & II showing epidermal and dermal component of skin. Connective tissue collagen appears green, muscle fibres appear red while red blood cells appear red

Discussion

Recent attention has been focused on the substitution of hematoxylin with locally available Hibiscus extract as nuclear stain in histopathology techniques. The extract solution was used progressively to stain lymph node and appendix with eosin as counter stain (Benard, 2008). It was recommended that it could substitute for H&E in the demonstration of general tissue structure. Later on, Egbujo and Colleagues (2008) used alum and iron mordanted solution of the extract to stain rat testis and recommended it as a nuclear stain. Years later, Benard and Colleagues (Benard *et al.*, 2015a) applied the extract solution on liver and kidney with Sorghum bicolor as counter stain and recommended that *S. bicolor* is an electrostatically compatible counter stain for *H. sabdariffa* nuclear staining.

In the same vein, the hibiscus extract solution was applied as nuclear stain for brain tissue (Benard *et al.*, 2015b) and hippocampus of rats (Benard *et al.*, 2016) with eosin, van Gieson and *S. bicolor* counter stains respectively. The

various workers recommended Hibiscus-eosin as H&E substitute for general tissue structure in brain, Hibiscus-van Gieson new staining technique in neuro-histology and Hibiscus-Sorghum as new morphological stain in neuro-histology respectively. Yetunde and Colleagues (2017) explored the staining ability of *H. sabdariffa* on appendix (lymphoid tissue) and concluded that the use of 50% extract concentration, pH 1.9 and temperature of 60°C gave desired result as a nuclear stain. Their findings also discouraged the use of mordant for *H. sabdariffa* staining. The present study used an iron mordanted *H. sabdariffa* extract solution as nuclear stain. In the work of Omorodion and Colleagues (2017), it was concluded that Roselle plant has the ability to stain tissue sections and staining intensity depended on the type of mordant used.

This present work attempts to find out the capability of Hibiscus-Trichrome staining technique as a substitute to the routinely used, hematoxylin- dependent, Masson's Trichrome

stain for connective tissue collagen, muscle and red blood cells differential staining.

Microscopical study revealed satisfactory staining by the new technique comparable with the standard Masson Trichrome (MT) (Fig I & II). Independent observers often mistook the Hibiscus-Trichrome for Masson Trichrome due to the superior clarity of the collagen fibers in the new technique. The good result recorded by the substitution testified to the flexibility and iso-electric compatibility of the Hibiscus extract solution as earlier reported (Muhammed et al., 2016) and trichrome counter stains. The new technique is cheaper, less cumbersome and faster.

References

- Al-Tikriti, S.A. and Walker, F. (1978). Anthocyanin BB: a nuclear stain substitute for haematoxylin. *Journal of Clinical Pathology*; 31:194-196.
- Bracegirdle, B. (1986). A History of Micro technique (2nd) Lincolnwood IL, Science Heritage LTD: 67.
- Benard, S.A. (2008). Iron-Roselle: A Progressive nuclear stain substitute for hematoxylin. *The Journal of Histotechnology*; 31(2):57-59.
- Benard, S.A., Muhammed, A.O., Fowotade, A.A., Afolabi, O.O., Olutunde, O.A. (2015a). Sorghum Bicolor Extract: A Suitable Counter Stain in Hibiscus Extract Nuclear Staining of Liver and Kidney. *African Journal of Cellular Pathology*; 4:13-16.
- Benard, S.A., Muhammed, A.O., Fowotade, A.A., Afolabi, O.O. and Olutunde, O.A. (2015b). Hibiscus Sabdariffa extract as Haematoxylin Substitute in the Histological Demonstration of Brain Tissues: *African Journal of Cellular Pathology*; 5: 32-35.
- Benard, S.A., Afolabi, O.O., Olutunde, O.A. (2016). Hibiscus Extract-Iron –Van Gieson: A new Morphological Staining Technique in Neuro-Histology. *African Journal of Cellular Pathology*; 30-34.
- Clark, G. and Kasten, F.H. (1983). History of Staining (3rd ed). Baltimore, Williams and Wilkins: 113.
- Egbujo, E.C., Adisa, O.J. and Yahaya, A.B. (2008). A Study of the Staining Effect of Roselle (Hibiscus Sabdariffa) on the Histologic Section of the Testis. *International Journal of Morphology*; 26(4): 927-930.
- Michael, T. (2009). Progress in the development of microscopic techniques for diagnostic pathology. *The Journal of Histotechnology*; 32(1): 9-19.
- Muhammed, A.O., Olutunde, O.A., Benard, S.A., Muhammad Ahmad, A.T. and Omoowo, B.T. (2016). Hibiscus-Shorgum: A New Morphological Stain in Neuro-Histology. *International Journal of Health Research and Innovation*; 4 (1): 2051-505.
- Omorodion, N.T. and Achukwu, P.U. (2017). Investigation of Hibiscus Sabdariffa (Roselle) as Histological Stain and in Assessment of Bar Bodies. *American Journal of Biomedical Science*; 9(1): 15-19.
- Sheehan, D.C. and Hrapchak, B.B. (1980). Theory and Practice of Histotechnology (2nd ed). St. Louis, MO.C.V. Mosby Company: 190.
- Yetunde, A.A., Ejike, E.C. and James, A.O. (2017). Study of the staining effect of Hibiscus sabdariffa in demonstrating the histologic features of the lymphoid tissues. *Bayero Journal of Medical Laboratory Science*; 2(2): 109-117.

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