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Hibiscus Iodine Staining of Cellular and Keratinized Tissues As A Nuclear Stain Substitute For Haematoxylin

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Abstract
An iodine-based formulation of a simple boiling water extract of Hibiscus sabdariffa was compounded and stored at 4°C. Formalin fixed paraffin embedded blocks of tissues from appendix, ovary, lymph node and skin were randomly retrieved from teaching block archives duly labelled and sectioned at five microns. Sections were stained with the Hibiscus iodine formulation in line with routine protocol for haematoxylin and eosin. Erhlich's haematoxylin was used as parallel control for each organ. Microscopic slide review by histopathology experts assessed the results as very good and revealed comparative staining with standard haematoxylin and eosin thus expanding the application of Hibiscus formulation in histopathological demonstration of cellular and keratinised tissues especially skin, lymph node, appendix and ovary.

Keywords: Roselle, Histology, Nucleus, Tissues, Hibiscus

Introduction

Cellular pathology Scientists, Pathologists, Histologists often depend on dyes to visualise cells and tissues. This could only be made possible through availability and access to reagents that form the backbone of histology techniques. While wealthy nations generally have access to these reagents, same cannot be said of low- and medium-income countries that have to contend with civil unrest, perennial wars, abject poverty, and devalued local currencies. In other to fill this huge gap, investigators in developing countries have turned to local sources of dyestuffs so as not to interrupt services.

In recent times, *Hibiscus sabdariffa* has been the most studied and foremost dye proposed for use in histology in developing countries especially in Africa and Nigeria to be specific. Typically, hibiscus extract has been used as a basis for dye formulations for local applications since it is not commercially available. This native dye has been applied for selective staining of cell nuclei to replace haematoxylin to meet critical need and resolve logistics issues.

Although haematoxylin is the most commonly used dye for histology, its history of interrupted supply, prohibitive high cost and unavailability (Benard, 2008; Dapson *et al*., 2010; Hayfaa and Richard, 2021a) justifies the search for local substitute that are readily available, easy to prepare and less harmful.

Unfortunately, other dyes recommended as haematoxylin substitutes are never available for workers in developing countries (Hayfaa and Richard, 2021a).

In Nigeria, *Hibiscus sabdariffa* is widely cultivated in the North West, North East, North Central and South West regions (Benard, 2018). The hibiscus formulation most common in literature has been iron based now termed, roseion (Benard, 2008; Benard *et al*., 2015a; Benard *et al*., 2015b; Muhammed *et al*., 2016; Benard *et al*., 2017; Agbede *et al*., 2017; Benard *et al*., 2018a; Benard *et al*., 2018b: Benard *et al*., 2019; and Olufunmilayo *et al*., 2020). This paper

reports the use of Roselle extract mordanted with iodine termed rosadine for the staining of lymph node, skin, ovary and appendix.

Methodology

Preparation of *H. sabdariffa* iodine solution

Ten grammes (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 millilitres (100ml) of the *H. sabdariffa* extract were mixed with 5g of sodium chloride, 1.2. ml of 10% anhydrous ferric chloride solution and 1.2 ml of Lugol's iodine. Storage was done at 4^0C.

Sectioning

Formalin fixed, paraffin embedded tissue blocks were retrieved from teaching archives and sectioned at three microns using a Leica Microtome. Lymph node sections were labelled A, Skin sections B, Appendix C and Ovary D. 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 iodine stain (rosadine) stained nuclei violet-blue to blue-black and cytoplasm, pink. Red blood cells stained red and collagen, pink. This is comparable to the staining by standard Harris haematoxylin.

Discussion

The histological use of *Hibiscus sabdariffa* as a nuclear stain has been critically reviewed in recent times and adduced to have merit (Hayfaa and Richard, 2021a) despite an earlier scepticism (Dapson *et al*., 2010). In their comprehensive critical review, the above authors proposed the plausibility of the application of Roselle as a nuclear stain substitute to haematoxylin while canvassing for more research in this direction. They also reported that earlier reports of two authors (Ebujo *et al*., 2008; Omorodion and Achukwu, 2017) on the use of Hibiscus as a biological stain were problematic for being ambiguous in photomicrographs clarification and inconsistent verbal description of results and photomicrographs respectively (Hayfaa and Richard, 2021a).

Earlier attempts were made to use *Hibiscus sabdariffa* extract for other purposes besides nuclear staining. Acidified extract in 70% alcohol was used to demonstrate all tissue components pink (Bassey *et al*., 2012). Other workers applied the extract to achieve staining results ranging from brownish red to red (Hashim, 2006, Ibnouf *et al*., 2014; Raheem *et al*., 2015; Ibnouf *et al*., 2016; Shridara *et al*., 2016). All these were applied on tissues. A potash alum 'hibiscus formulation was also applied on fungi in culture which was stained red (Abubakar *et al*., 2012).

Recently, a soaking method of extraction was used at room temperature to prepare a Roselle based staining solution that contain 3% hydrogen peroxide and phosphomolybdic acid as solution A and B. Equal volumes of stock solution A and B were used and differentiated with 2% aqueous picric acid (Hayfaa and Richard, 2021a). The staining was nuclei specific to replace haematoxylin.

This author and co-workers have used iron-based Roselle formulations derived from a simple boiling water extract of *Hibiscus sabdariffa* mordanted with ferric chloride. The achieved objective was for nuclei specific staining (Benard, 2008; Benard *et al*., 2015a; Benard *et al*., 2015b; Muhammed *et al*., 2016; Benard *et al*., 2017; Agbede *et al*., 2017; Benard

et al., 2018a; Benard *et al*., 2018b: Benard *et al*., 2019 and Olufunmilayo *et al*., 2020). The use of hibiscus formulations for selective nuclei staining has now become scientifically validated and authenticated (Hayfaa and Richard, 2021a; Hayfaa and Richard, 2021b).

In this work, iodine was used as mordant. The chemical basis of hibiscus staining and combination with metals as mordants have been adduced to molecular stabilization and complexation whereby at lower pH, protonation occurs while at pH of ≤ 3 deprotonation occurs. The colour of the nuclei at this pH is violet. Hibiscus staining of nuclei with violet colour has been reported to fall between the pH of 2.8-3.0 (Hayfaa and Richard, 2021a).

In this work, glacial acetic acid was replaced with Lugol's iodine which significantly improved staining contrast observed in nuclear and cytoplasmic components. Glacial acetic acid had been earlier reported to interfere with eosin counterstain in hibiscus formulations (Benard *et al*., 2018; Egbujo *et al*., 2008). In other to rule out the effect of the ferric iron compounded with our formulation as a probable confounding factor, parallel staining was done with iron-Roselle formulation. The result from the experiment showed that it was inferior to the rosadine therefore the iron Roselle couldn't have been responsible for the very good result observed in the organs.

In this work, cellular components were well demonstrated in lymph nodes, skin, ovary and appendix (Fig 1-3). The keratin layer of the skin, epidermis, and melanin pigment were also well demonstrated (Fig 4). The staining quality observed in the nuclei, cytoplasm, adipose tissue, red blood cells, keratin, dermis, and melanin pigment was very good following independent assessment by histopathology experts.

Most routine haematoxylin stains are regressive. This work applied the rosadine formulation as a regressive stain with impressive outcome. This gives hope for its application in routine histopathology laboratories especially in resource limited countries.

This work has demonstrated the versatility of hibiscus formulation in demonstrating cellular components of tissues and organs in a comparable manner to standard haematoxylin. The Roselle-iodine formulation, rosadine has been successfully applied regressively with very good outcome especially in the demonstration of highly cellular tissues such as lymph node, appendix and ovary. Observation for keratinised skin is similar. It is therefore recommended for use as histology and biological stain to replace haematoxylin in nuclear staining for its ease of preparation, local availability, eco-friendliness, and safety.

References

Abubakar, S., Usman, A.B., Etim, V., Nnadi, O, Alaku, C. (2012). Application of organic dyes from Roselle calyx (Hibiscus sabdariffa Linn.) for mycological staining. *International journal of Innovation and Development*; **1**: 687-690.

Bassey, R.B., Bakare, A.A., Peter, A.I., Oremosu, A.A.(2012). Factors influencing extract of Hibiscus sabdariffa staining, off rat testes. *Biotechnic and Histochemistry*; **87**: 403-407.

Benard, S.A., Adeniyi, T.D., Bankole, J.K., Okoye, J.O., (2018). Histomorphological Staining Of Selected Organs By Iron-Roselle. *Advances in Medicine and Medical Research*; **28(5)**: 1-12.

Benard, S.A. (2008). Iron-Roselle, A Progressive Nuclear Stain Substitute for Haematoxylin*;* *The Journal of Histotechnology*; **31(2)**: 57-59.

Benard, S.A. (2021). A study on the nuclear staining of hibiscus sabdariffa aqueous extract (ZOBO) mordanted with ferric chloride on the connective tissues of selected organs. A Fellowship thesis submitted to the Medical Laboratory Science Council of Nigeria.

Benard SA, Fowotade AA, Olutunde AO, Afolabi OO (2019). Hibiscus-Trichrome Stain for Collagen, Muscle And Red Blood Cells in Skin Tissue. *Sokoto Journal of Medical Laboratory Science*; **4(1)**: 39-42.

Benard S.A**.**, Afolabi O.O., Bankole, J.K., Okoye, JO, Fowotade, A.A., Olutunde, O.A. (2018). The Determination of Optimum Staining Time For Hibiscus Extract Nuclear Staining Using Buffered Formalin Fixed,

Paraffin Embedded Lung Tissue. *Sokoto Journal of Medical Laboratory Science*; **3(2)**: 82-85.

Benard, S.A., Adeniyi, T.D., Bankole, J.K., Okoye, J.O.(2018). Histomorphological Staining Of Selected Organs By Iron-Roselle. *Advances in Medicine and Medical Research*; **28(5)**: 1-12. DOI:10.9734/JAMMR/2018/42802

Benard, S.A., Muhammed, A.O., Fowotade, A.A., Afolabi, O.O., Olutunde, O.A. (2015). Hibiscus sabdariffa extract as haematoxylin substitute in the histological demonstration of brain tissues. *African Journal of Cellular Pathology*; **35**: 32-35.

Benard, S.A., Muhammed, A.O., Nwakpu, S., Fowotade, A.A., Afolabi. O.O., Olutunde, O.A. (2015). Sorghum bicolour extract: A Suitable Counter Stain in Hibiscus Extract Nuclear Staining of Liver and Kidney. *African Journal of Cellular Pathology*; **4**:13-16.

Dapson, R.W., Horobin, R.W., Kiernan, J. (2010). Haematoxylin shortages: their causes and duration, and other dyes that can replace hemalum in routine haematoxylin and eosin staining. *Biotechnic and Histochemistry*; **85**: 55-63.

Egbujo, E.C., Adisa, O.J., Yahaya, A.B. (2008). A study of the staining effect of Roselle

(Hibiscus sabdariffa) on the histological section of the testis; *International Journal of Morphology;* **26(4)**: 927-930

Hashim, E.A. (2006). The use of watery extract of Kujarat flowers *Hibiscus sabdarifa* as a natural histological stain. *Iraqi Journal of Medical Science*; **5**: 29-33.

Hayfaa, A., and Richard, W.D. (2021a). Use of Roselle extracted from *Hibiscus sabdariffa* for histological staining: a critical review and rational stain formulation. Biotechnic and Histochemistry; **96(2)**: 94-101. DOI: 10.1080/10520295.2020.1769864.

Hayfaa, A., and Richard, W.D. (2021b). Molecular stabilization and complexation: the secrets of making a nuclear-selective histological stain from naturally occurring anthocyanins without oxidation. *Biotechnic and Histochemistry*: 95-102. DOI: 10.1080/10520295. 2021.1881617.

Ibnouf, A.A., AbudlRaheem, E., SeedAhmed, M., Dahab, D. (2014). Assessment of staining quality of Roselle (Hibiscus sabdariffa) on formalin-fixed paraffin-embedded renal tissue sections. *International Journal of Current Research and Review*; **6**: 26-28.

Ibnouf, A-A.O., Raheem, E.M.A., Fdl-Elmula, I. (2016). Using Hibiscus sabdariffa in staining of appendicular tissue sections. *European Journal of Biomedical and Pharmaceutical Science*; **3**: 16-18.

Muhammed, A.AO., Olutunde, O.A., Benard, S.A., Muhammad, A.T., Omoowo, B.T. (2016). Hibiscus 'sorghum: a new morphological staining neuro-histology. International *Journal of Health Research and Innovation*; **4**: 31-38.

Olufunmilayo, O.A., Benard S.A., Balogun, M.O., Fowotade, A.A., Olutunde, O.A., Afolabi, O.O. (2020). Regressive Hibiscus sabdariffa Extract Nuclear Stain Using Formalin Fixed Paraffin Embedded Skin, Lymph node and Appendix. *Sokoto Journal of Medical Laboratory Science*; **5(2)**: 40-44.

Omorodion, N.T., Achukwu, P.U. (2017). Investigation of Hibiscus sabdariffa (Roselle) as histological stain and in assessment of Bar bodies. *American Journal of Biomedical Science*; **9**:15-19.

Raheem, E.M.A., Ibnouf, A.O., Shingeray, O.H., Farah, H.J.M.(2015). Use of Hibiscus sabdariffa extract as a natural histological stain of the skin. American of Research Communication; **3**: 211-216.

Sridhara, S.U., Raju, S., Gopalkrishna, A.H., Haragannavar, V.C. (2016). Hibiscus: a different hue in histopathology. *Journal of Medical Radiology and Pathological Surgery*; **2**:9-12.

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

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

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

Figures

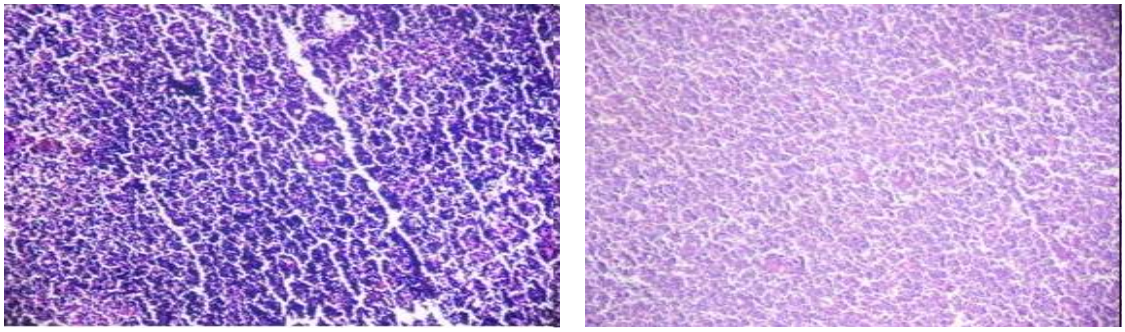

Fig. 1: Lymph node stained with Rosadine showing lymphocytes, macrophages and sinusoids comparable with standard H&E X100

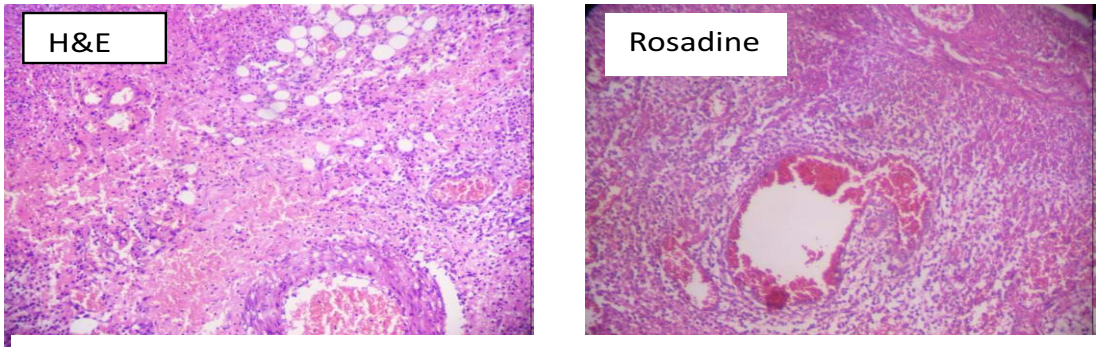

Fig. 2: Appendix stained with Rosadine showing germinal cells, red blood cells, and blood vessels comparable with standard H&E X100

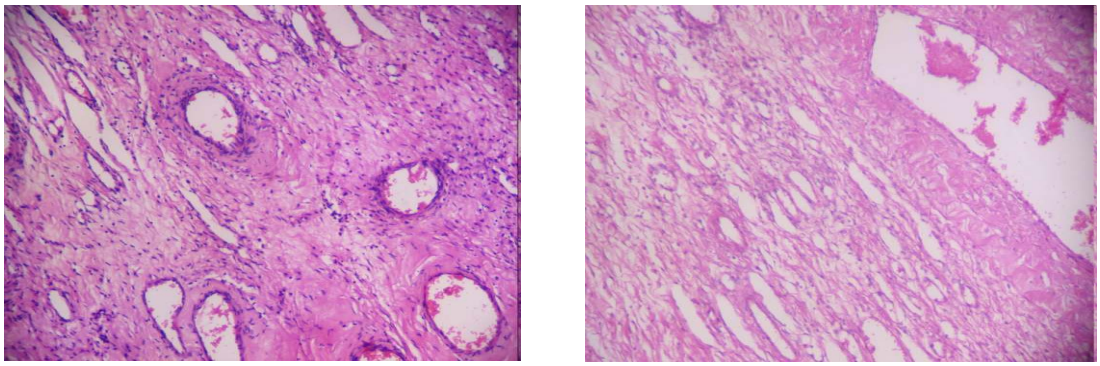

Fig. 3: Ovary stained with Rosadine showing red blood cells, blood vessels and connective tissues comparable with standard H&E X100

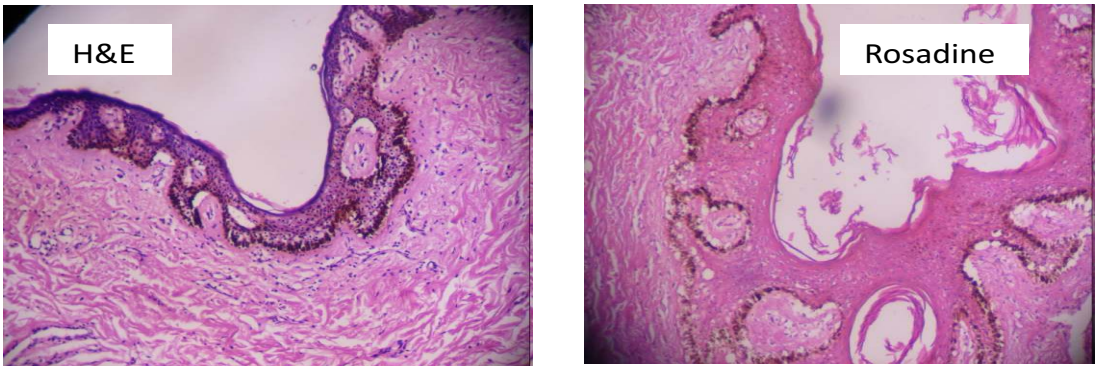

Fig. 4: Skin stained with rosadine showing pigments, epidermis and keratin comparable with standard H&E X100

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