ASSESSMENT OF THE OXIDATIVE POTENTIAL OF *CITRUS LIMON, CITRUS AURANTIFOLIA* JUICE EXTRACTS AND COUNTERSTAINING PROSPECTS OF *HIBISCUS SABDARIFFA* LEAF EXTRACT TO THE CONVENTIONAL PERIODIC ACID SCHIFF'S TECHNIQUE

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Received: 2nd September, 2024 **Accepted**: 15th December, 2024 **Published**: 31st December, 2024

ABSTRACT
Background: Periodic acid Schiff (PAS) is a widely used histochemical staining technique specifically for the demonstration of carbohydrate molecules in tissues. *Citrus limon* and *Citrus aurantifolia* juice are non-toxic juices that are safe for consumption and do not cause any adverse effects when exposed to bodily tissues.
Aim: This study demonstrates the oxidative potential of *Citrus limon* and *Citrus aurantifolia* juice as a substitute for periodic acid in the conventional PAS staining technique while also evaluating the counterstaining quality of *Hibiscus sabdariffa* extract.
Methodology: Histological sections were obtained from archival blocks retrieved from the histopathology service laboratory of Bowen University Teaching Hospital and stained using the conventional Periodic acid Schiff technique while modification in staining protocol was carried out on subsequent slides that were treated with fresh juice extracts of *Citrus limon* and *Citrus aurantifolia* and extracts of *Hibiscus sabdariffa* obtained from its calyces. While the control tissue sections were stained with the conventional PAS technique, other sections of tissues were independently treated by oxidizing with *Citrus limon* or *Citrus aurantifolia* juice respectively and continued with Schiff's reagent while counterstaining with Harris hematoxylin. The other tissue sections were treated with *Citrus limon* Schiff and *Citrus aurantifolia* Schiff technique by first oxidizing with *Citrus limon* juice / *Citrus aurantifolia* juice, treated with the Schiff's reagent and followed by counterstaining with *Hibiscus sabdariffa* extract.
Results: The result showed that the periodic acid Schiff, *Citrus limon* Schiff and *Citrus aurantifolia* Schiff-stained liver and kidney sections appear similarly with purple-magenta colour which demonstrates the glycogen and glycoprotein content of the tissues, as well as the blue colouration demonstrating the counterstained nuclei.
Conclusion: This study showed that the substitution of the 1% periodic acid with freshly prepared *Citrus limon* or *Citrus aurantifolia* juice and counterstaining with the extract of *Hibiscus sabdariffa* impacted a staining effect similar to the conventional PAS staining protocol thus suggesting it as a possible substitute that is cost-effective and readily available alternative in the demonstration of carbohydrates in histologic tissue.
Keywords: Histochemistry, special stains, periodic acid Schiff, *Citrus plant*.

INTRODUCTION

Histological staining is a fundamental technique in the field of histology and it plays a pivotal role in the examination of biological tissues, allowing for the visualization and differentiation of diverse cellular and structural components (Weiss *et al*., 2017).

Citation: Adeniyi, T. D., Moronkeji, A., Abdulsalam, J. A., Oluyemi, T. O. and Adejugbe, H. A. (2024): Assessment Of The Oxidative Potential Of *Citrus Limon*, *Citrus Aurantifolia* Juice Extracts And Counterstaining Prospects Of *Hibiscus Sabdariffa* Leaf Extract To The Conventional Periodic Acid Schiff's Technique *BJMLS 9(2): 129 - 139*

The Periodic Acid Schiff (PAS) reaction is a commonly used histochemical technique that was initially proposed by Shabadash in 1946 as a method to stain glycogen, even though the majority of literature and publications on the subject give credit to Hotchkiss McManus (Laga, 2020). This staining technique uses periodic acid, also known as iodic (VII) acid, a type of iodine oxoacid with the chemical formula HIO_4, H_5IO_6 or H_3IO_6. Periodic acid may exist in three forms; metaperiodate (HIO_4), orthoperiodate (H_5IO_6), and paraperiodate (H_3IO_6) (Arndt *et al*., 2022). While orthoperiodic acid (H_5IO_6) is present in high concentrations, it dissociates into hydronium and orthoperiodate ions in a diluted aqueous solution (Zhdankin, 2021). Periodic acid is pronounced "per-iodic" rather than "periodic" since it is not derived from the word "period" but from the word "iodine": per-iodic acid (as described iodic acid, perchloric acid) (Gogia *et al*., 2021). With orthoperiodic acid (ortho - more water) and metaperiodic acid (meta - less water), two forms of periodate salts can be formed. Periodic acid is used as an oxidising agent in many chemical activities. The adverse effects of periodic acid exposure are mostly acute, with the immediate consequence of exposure being burns, inflammation, and oedema, as well as having a damaging effect on mucosal surfaces and the upper respiratory tract. Long-term tissue damage from the corrosive element is of greater concern (Gad and Gad, 2014; Song *et al*., 2023). Although it is classified as a weak acid, periodic acid is a relatively powerful oxidizer due to its strong corrosive nature when concentrated and its ability to quickly oxidize bodily tissues, it is a powerful lachrymator (Gad and Gad, 2014).

Citrus limon, also known as lemon, is a member of the Rutaceae family of flowering plants of little evergreen trees (Klimek-Szczykutowicz and Szopa, 2020). Some of the names of *Citrus limon* in several languages include lemon (English), *Zitrone* (German), *le citron* (French), *limón* (Spanish), and *níngméng* (Chinese). Lemon is the most popular name for this fruit, which can be eaten and it is the second-most significant species of citrus fruit after orange, which is grown over 4.4 million tonnes annually (Makni *et al*., 2018). Lemon contains significant amounts of highly important natural chemicals such as beta- and gamma-sitosterol, ascorbic acid, minerals, citric acid, essential oils, phenolic compounds, polyethoxylated chemicals, and flavonoids (Makni *et al*., 2018; Rafique *et al*., 2020). Avon, Berna, Baboon, Bears, and Cameron are a few of the numerous varieties of lemon (Mshelia *et al*., 2018). Previous studies focused more on the use of lime juice to replace periodic acid in periodic acid Schiff stains due to its toxic nature.

The historical use of lime juice as a staining agent can be traced back to a time when the availability of laboratory chemicals was limited. In the early days of histological research, scientists and histologists often had to rely on readily accessible natural substances to aid in their studies. Another member of the Rutaceae family of flowering plants, commonly known as the *Citrus aurantifolia*, is usually placed in the order Sapindale (Khalil *et al*., 2024). The fruit is usually about 3 to 4 cm (1 to 1.5 inches) in diameter, oval in shape, often with a small apical nipple, and the peel is thin and greenish yellow when the fruit is ripe. The pulp is tender, juicy, yellowish-green in colour and acidic (Abdou, 2023). Lime juice, with its acidic properties and unique chemical composition, was one such substance that may have been considered for staining purposes (Jones and Brown, 2015).

Hematoxylin is the most commonly used nuclear counterstain in histology that is extracted from the heartwood of the logwood tree *Haematoxylum campechianum* that belongs to the order *Legumiosae* (Genus-*Eucaesalpiniea*), which grows in Campeche, Mexico (Mohandas *et al*., 2019).

Hematoxylin comes in many types, but *H. campechianum* produces the best-looking coloured wood and it is the most widely used nuclear stain due to its durability, simplicity in distinction, and relative permanence (Ortiz-Hidalgo and Pina-Oviedo, 2018; Mohandas *et al*., 2019; Mahapatra *et al*., 2020). Natural dyes can be derived from a variety of plant parts, including leaves, fruits, seeds, flowers, barks, and roots (Izquierdo-Vega *et al*., 2020). The creation of substitute organic and environmentally acceptable dyes from these natural sources has been prompted by the global shortage of hematoxylin and the harmful consequences of chemicals and synthetic dyes like eosin. As humanity's awareness of the environment has risen, using these non-allergic, non-toxic, and biodegradable stains has become essential (Mohandas *et al*., 2019; Izquierdo-Vega *et al*., 2020). *Hibiscus sabdariffa* (Hs), often known as roselle, is a crop that is ideal for developing nations because it is reasonably simple to grow. It can be grown as part of multi-cropping systems and can be used as both food and fibre (Da-Costa-Rocha *et al*., 2014). It is commonly grown in tropical and subtropical areas including India, Saudi Arabia, China, Malaysia, Indonesia, Philippines, Vietnam, Sudan, Egypt, Nigeria, and México (Izquierdo-Vega *et al*., 2020). There are two main types of *Hibiscus sabdariffa*: Hibiscus sabdariffa var. altissima Wester, which is grown for its fibre resembling jute, and *Hibiscus sabdariffa* var. sabdariffa. (Da-Costa-Rocha *et al*., 2014). Roselle produces red edible calyxes that can be used to make a variety of delicious items (Bule *et al*., 2020). The calyces of *H. sabdariffa* (cHs), which can be fresh or dried, are used to make herbal drinks, hot and cold beverages, fermented beverages, wine, jam, jellied confections, ice cream, chocolates, flavourings, puddings, and cakes (Izquierdo-Vega *et al*., 2020). The most significant source of anthocyanins, which give various plant components their colour, is *Hibiscus sabdariffa* (Izquierdo-Vega *et al*., 2020). The ideal pH range for staining *H. sabdariffa* extract is between 2.5 and 4.0, and its colour ranges from red to purple to blue. The *Hibiscus sabdariffa* extract has a deep red colour that dissolves in water, turns pink when HCL is added, and turns bluish-green when alkali is added (Benard *et al*., 2015). This study evaluated the oxidative potential of *Citrus limon* and *Citrus aurantifolia* juice as an efficient replacement for 1% periodic acid, as well as extracts from *Hibiscus sabdariffa* as a substitute for Haematoxylin as a counterstain in the traditional PAS methodology for glycogen demonstration.

MATERIALS AND METHODS

Study Area

This study was carried out at the Department of Medical Laboratory Science Research Facility, Bowen University, Iwo, Osun State, Nigeria.

Preparation of Extracts

Preparation of Hibiscus sabdariffa extract Solution

Dry calyces of *Hibiscus sabdariffa* were purchased in Sabo, a local market in Ogbomoso, Oyo State, Nigeria and processed as recommended by Benard, (2008). The calyces were authenticated by a Botanist in the Department of Biochemistry, Bowen University Iwo, Osun State, Nigeria, and assigned the authentication number BUH 040. The dry calyces of *Hibiscus sabdariffa* were ground using a Binatone blender to a fairly powdery form. To 30g of the ground red calyces of *Hibiscus sabdariffa* in a conical flask, 600ml of distilled water was added and boiled to give the brilliant red coloured extract which was immediately allowed to cool and was spun at 3,000 rpm for 20 minutes, and then 5,000 rpm for 10 minutes.

The supernatant was then filtered to give a pure *Hibiscus sabdariffa* extract and the pH was measured using a pH meter. The staining formula was compounded afterwards. To 400ml of the pure *Hibiscus sabdariffa* extract, 20g of NaCl, 4.8ml of 10% ferric chloride solution, and 12ml of Glacial acetic acid were added.

Extraction of Lemon Juice

Citrus limon and *Citrus aurantifolia* fruits that were plucked within the school premises were authenticated by a Botanist in the Department of Biochemistry, Bowen University Iwo, Osun State, Nigeria, and were assigned the authentication number BUH 038 and BUH 039 respectively. The rinds were peeled off and sliced into two halves and the juices were extracted manually by squeezing followed by further filtering through a four-layer fold of sterile gauze and then spun twice at 3000 rpm and 5000 rpm for a duration of twenty and ten minutes respectively.

pH measurement of extracts.

The pH of the juice extract of *Citrus limon* and *Citrus aurantifolia* was measured and a pH value of 2.5 and 2.6 were respectively obtained while the pH value for *Hibiscus sabdariffa* was confirmed to be 3.3 using a digital pH meter (Apera Instrument P20).

Ethical consideration

Ethical approval was obtained from the Bowen University Teaching Hospital Research ethics committee and assigned approval number BUTH/REC-967 on the 26th of September 2023.

Histopathological Studies

Archival liver and kidney samples from the histopathology service laboratory, Bowen University Teaching Hospital, Iwo, Nigeria (BUTH) were sectioned at 4 µm thickness using a semi-automatic ERM 3100 Hestion Histology rotary microtome and grouped accordingly. The paraffin wax processed tissue sections were divided into four (4) groups. The control section in the first group was oxidized with Periodic acid, then treated with Schiff's reagent and counterstained with Harris Haematoxylin as described by Okobi *et al*. (2023). The second group of the tissue sections were oxidized with freshly prepared *Citrus limon* juice at pH 2.5 then continued with Schiff's reagent and then counterstained with Harris Haematoxylin. The third group of the tissue section were oxidized with freshly prepared *Citrus limon* juice extracts at pH 2.5 and then followed by counterstaining with the extract of *Hibiscus sabdariffa*. The fourth group of the tissue section were oxidized with freshly prepared *Citrus aurantifolia* juice extracts at pH 2.6 and then followed by counterstaining with the extract of *Hibiscus sabdariffa*. The stained slides across all the groups were analyzed with the aid of a light microscope (Moronkeji *et al.,* 2024).

Standard Procedure of Staining for PAS (Control)

The control slides were stained using the standard PAS staining protocol as described by Okobi *et al*. (2023). Briefly, the slides were dewaxed and hydrated in descending grades of alcohol and transferred to water followed by oxidizing using 1% periodic acid at pH 2.5 for 10 minutes. The sections were rinsed with water followed by treatment with Schiff's reagent for 30 minutes. The sections were rinsed in water and counterstained using Harris Haematoxylin at pH 3.1 for 2 minutes after which sections were briefly differentiated with 1% acid alcohol and rinsed immediately and blued in tap water for 10 minutes and followed with dehydration in ascending grades of alcohol, cleared in two changes of xylene and mounted using DPX.

Modification of PAS protocol with Citrus limon juice oxidation

The tissue sections were dewaxed and hydrated followed by oxidizing with *Citrus limon* juice at pH 2.5 for 10 minutes after which sections were thoroughly rinsed in tap water and treated with the Schiff's solution for 30 minutes. The treated sections were counterstained with *Hibiscus sabdariffa* extract at pH 3.3 for 2 minutes followed by a brief differentiation with 1% acid alcohol after which bluing was ensured for 10 minutes using Tap water. The sections were further dehydrated in ascending grades of alcohol followed by clearing in two changes of Xylene and mounted using DPX.

Modification of PAS protocol with a co-treatment with Citrus limon and Citrus aurantifolia juice Tissue sections were dewaxed and hydrated followed by oxidization with *Citrus limon* juice at pH 2.5 for 10 minutes and further treatment with juice extract of *Citrus aurantifolia* juice at pH 2.6 for 10 minutes. The sections were thoroughly rinsed in water and treated with Schiff's solution for 30 minutes after which they were rinsed and counterstained with *Hibiscus sabdariffa* extract at pH 3.3 for 2 minutes followed with a brief differentiation using 1% acid alcohol. Tissue sections were blued in tap water for 10 minutes and dehydrated using ascending grades of alcohol after which sections were cleared in Xylene and mounted using DPX.

RESULTS

Histological studies

Histological findings in this study indicated that the periodic acid Schiff, *Citrus limon* Schiff and *Citrus aurantifolia* Schiff-stained tissues were similar to the conventional PAS technique producing a purple-magenta colouration. The freshly prepared *Citrus limon* and *Citrus aurantifolia* juice cause the oxidation of 1, 2-glycol bonds in the glycogen and glycoprotein molecules of the tissues to result in the formation of aldehyde groups which then react with Schiff's reagent to produce a chromogen, magenta. The extract of *Hibiscus sabdariffa* also stained the nucleus blue as compared to hematoxylin. All the slides show similar staining characteristics with the nucleus blue and carbohydrate molecules magenta in the stained liver and Kidney tissue.

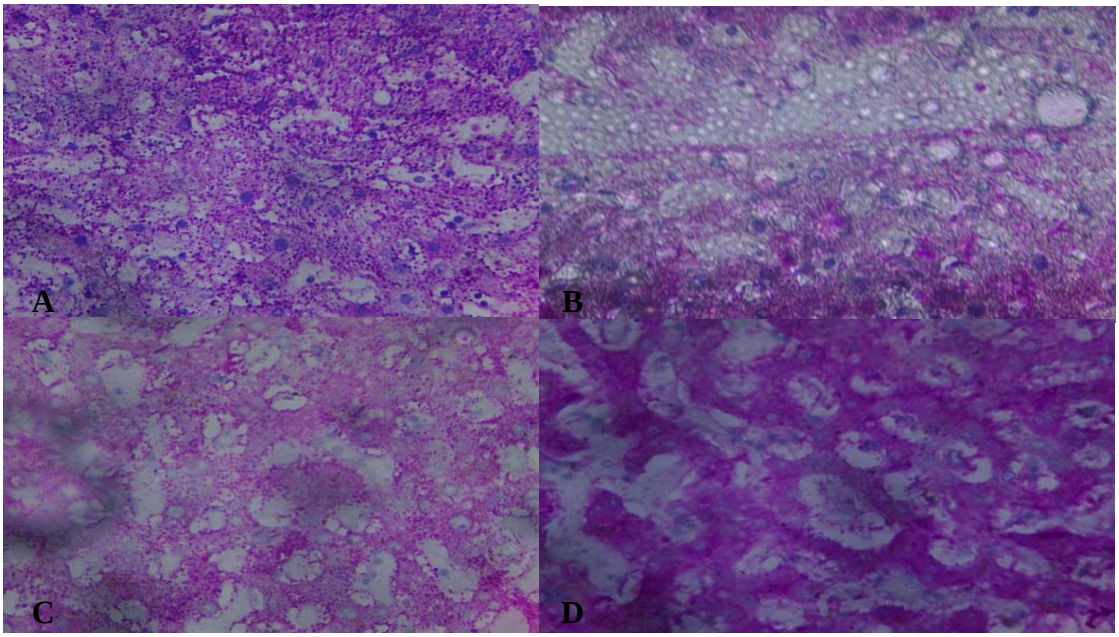

Figure 1. a. Control liver samples stained with conventional PAS technique. b. Liver section oxidized with *Citrus limon* juice and counterstained with Harris hematoxylin. c. Liver section oxidized with *Citrus limon* juice and counterstained with *Hibiscus sabdariffa* extract. d. liver section oxidized with *Citrus aurantifolia* juice and counterstained with *Hibiscus sabdariffa* extract.

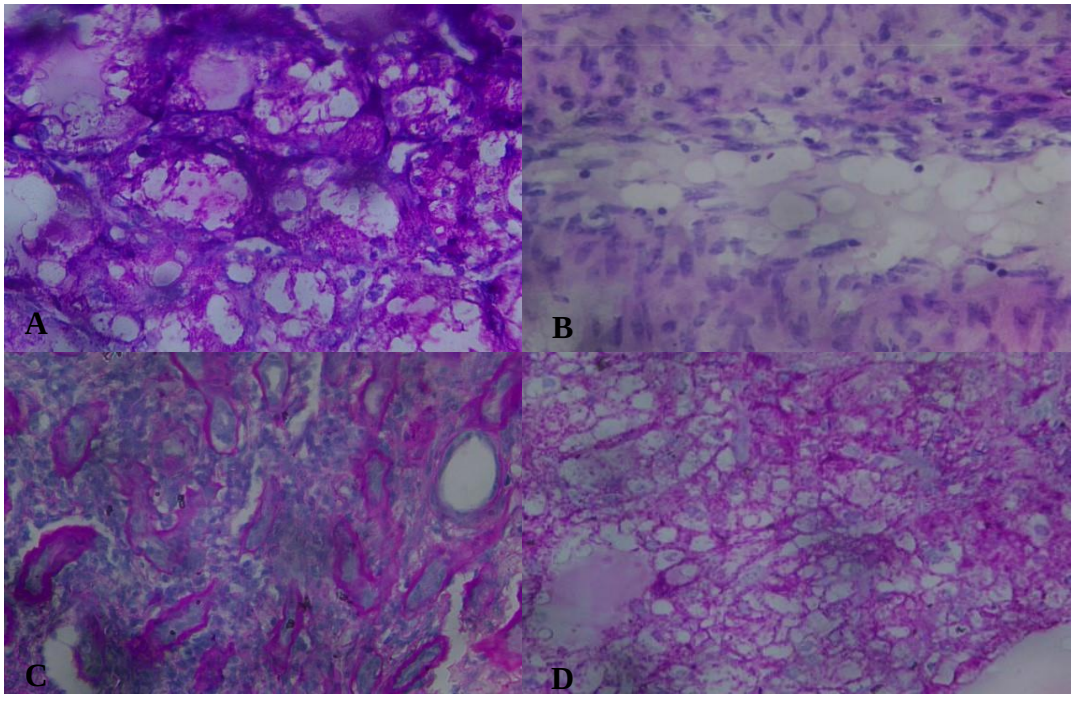

Figure 2. **a.** Control kidney sections stained with the conventional PAS technique **b.** Kidney section oxidized with *Citrus limon* juice and counterstained with Harris hematoxylin. **c.** Kidney section oxidized with *Citrus limon* juice and counterstained with *Hibiscus sabdariffa* extract. **d.** kidney section oxidized with *Citrus aurantifolia* juice and counterstained with *Hibiscus sabdariffa* extract.

DISCUSSION

Histological staining is an extremely important part of disease diagnosis. The Periodic Acid Schiff staining method is still commonly employed in histochemistry to detect glycogen in organs such as the liver when glycogen storage disorders are suspected. Furthermore, several studies have investigated the staining capacity of different plants to act as substitutes for certain standardised histological staining techniques (Ngokere *et al*., 2016; Steinke *et al*., 2018; Song *et al*., 2023; Osman *et al*., 2023). Reports by Benard, (2008) and Osman *et al*. (2023) documented the potential of *Hibiscus sabdariffa* extract as a viable nuclear stain that may effectively replace haematoxylin in various histological staining methods with reports stating its ability to stain basophilic tissue components such as the lumen of seminiferous tubules and spermatogenic cells (Bassey *et al*., 2012). Ma'aruf *et al.* (2020a; 2020b) also demonstrated the staining potential of *Hibiscus sabdariffa* in the demonstration of microorganisms such as fungi and bacteria in tissue sections, further indicating its suitability in formalin-fixed, paraffin-embedded tissue sections for diagnostic bacteriology and morphological identification of fungi. Reports have also indicated that the use of *Hibiscus sabdariffa* extract when not oxidised or mordanted resulted in all tissue components staining pink with the modified Haematoxylin and Eosin (H&E) staining procedure (Raheem *et al*., 2015; Ibnouf *et al*., 2016). However, when oxidized and mordanted, staining with *Hibiscus sabdariffa* and Eosin yields results comparable to the conventional H&E staining protocol (Bernard, 2015; Muhammed *et al*., 2016; Osman *et al*., 2023). Additional studies have also indicated that other oxidizing agents can also aid in the oxidation of carbohydrates besides periodic acid, despite being the most widely utilized and possibly the most successful (Rooshenass *et al*., 2017; Okorie *et al*., 2018; Louis *et al*., 2019). In this study, we observed that the modification of the PAS staining technique by replacing the 1% periodic acid with *Citrus limon* and *Citrus aurantifolia* juice and counterstaining with *Hibiscus sabdariffa* were similar to the conventional PAS technique. The freshly prepared *Citrus limon* juice and *Citrus aurantifolia* juice causes the oxidation of 1, 2-glycol bonds in the glycogen and glycoprotein molecules of the tissues to result in the formation of aldehyde groups which then react with Schiff's reagent to produce a chromogen, magenta, similarly as periodic acid in the conventional PAS technique (Ngokere *et al*., 2016; Steinke *et al*., 2018; Song *et al*., 2023). The extract of *Hibiscus sabdariffa* also stained the nucleus blue as compared to hematoxylin. Given the excellent staining reaction that lemon and lime juices provide when employed as an oxidizing agent, as well as considering their little or no toxicity and cost benefits, it is suggested that the oxidizing potential can be harnessed in this histochemical technique. The sections oxidized with juices unquestionably stained more intensely with Schiff solution, revealing the presence of carbohydrates and other structures that were first oxidized by the juices, as demonstrated by the nuclei's blue colouration in the kidney and liver tissue sections as well as their magenta colouration. The lemon and lime juice Schiff staining technique has been used in conjunction with periodic acid-positive materials, as established by Culling (1963) and Pearse (1972). However, the use of these juices, as well as the staining potential of *Hibiscus sabdariffa*, in the PAS protocol has not been established.

Studies have shown that the lemon and lime juice Schiff's technique revealed a list of periodic acid-positive materials, including the basement membrane, Bowman's capsule, and glomerular capillaries, as documented by Culling (1963), Luciano and Moeckel (2019), De Haan *et al*, (2021), Kotob *et al.* (2021), and Okobi *et al*. (2023).

Our findings in this study are consistent with the reports of Fasogbon et al. (2018), who documented that lime-Schiff stained the liver and brain in the same way that periodic acid Schiff did, producing a purple-magenta hue. Furthermore, Bernard, (2008) documented the staining capability of *Hibiscus sabdariffa*. This study documented the role of *Citrus limon* and *Citrus aurantifolia* in the oxidation reaction of 1, 2-glycol bonds of carbohydrate molecules in the studied tissues to produce aldehydes with *Hibiscus sabdariffa* extract serving as a quality nuclear-staining reaction that can serve as a substitute for hematoxylin in the conventional periodic acid Schiff technique.

CONCLUSION

Citrus limon Schiff and *Citrus aurantifolia* Schiff-stained tissues, comparable to the periodic acid Schiff procedure, produced a purple magenta hue, with *Hibiscus sabdariffa* acting as a nuclei counterstain, similar to Haematoxylin. Furthermore, given their low or no toxicity, as well as their cost-effectiveness and availability, the plants studied can be used as prospective alternatives in the demonstration of carbohydrates using the modified PAS approach. More study is needed to focus on developing more natural refined products with longer shelf lives, as well as replacing them with harmful compounds where suitable in other histopathological staining procedures.

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