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Modification of Periodic Acid Schiff and Haematoxylin Van Geison Solutions with Ethanol and Ethyl Acetate Wood Extract of *Erythroxylum*

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Abstract: Background: In histopathology, stains are substances or biological dyes, which colour tissues in order to aid optical differentiation of tissue component. Erythroxylum (erythroxylon) also known as redheart wood, is a genus of tropical flowering plants in the family Erythroxylacae, It has been observed that it has bright-red color when freshly cut that darkens to deep red over time. Aim: The aim of this study is to determine the staining reaction and novel modification of periodic acid schiff and Haematoxylin Van Gieson solutions with ethanoic and ethylacetate wood extract of erythroxylum plant on kidney, liver, lung and intestinal tissue section of Rattus norvegicus as an alternative to Schiff reagent, compared with haematoxylin and eosin staining, Weigert's iron haematoxylin Van gieson and Periodic acid Schiff staining method as control. Materials and Methods: Three serial sections were obtained from a block of a histological processed lung tissue of Rattus norvegicus and labelled A to C. Section A served as controls. Section As' were stained with Haematoxylin and Eosin staining, Weigert's Haematoxylin and Van Gieson Staining and Periodic acid Schiff reagent methods as controls. Test section Bs'were stained using Harris haematoxylin, Weigert's Iron Haematoxylin, Periodic Acid and counter stained with Ethanol extract of erythroxylum solutions respectively. Tests Section Cs' were all treated with the primary stained or oxidized with the required periodic acid and counter stained with ethylacetate extract of erthroxylum. Results: Liver sections treated with Periodic acid and counter stained with ethanol erythroxylum extract and ethylacetate erythroxylum solutions all revealed a comparable hepatic details as seen in periodic acid schiff solution. Similarly kidney sections of Rattus norvegicus stained with Weigert haematoxylin and counter stained with ethanol erythroxylum extract and ethylacetate erythroxylum extract solution also shown a comparable and better optical differentiation of the tubular structures and podocytes. Erythroxylum is a promising histological stain which is natural and readily available. Conclusion: The dye may serve in the demonstration of tissue constituents and serve as a useful cytoplasmic stain to replace other dyes for histological diagnosis of diseases.

Keywords: Dyes, *Erythroxylum*, Extract, Haematoxylin, Stain, Periodic acid

Introduction

Erythroxylum (erythroxylon) also known as redheart wood, is a genus of tropical flowering plants in the family Erythroxylacae [1]. Erythroxylum species were used by indigenous people of South America long before the domestication of plants. Few species such as *E. coca* have been utilized for thousands of years specifically for their tropane alkaloid content [2]. It has also been observed that it has bright-red color when freshly cut that darkens to deep red over time perhaps following natural oxidation of the hue [3].

Erythroxylum is currently gaining attention globally following its medicinal, nutritional, agricultural, and cosmetic values by several countries and scientific groups. In addition, with the ever-increasing capabilities of chemical and genetic analyses, wild species of Erythroxylum are being described and subjected to new studies identifying their potential for future development of medicines [4].

In histopathology, stains are substances or biological dyes, which colour tissues in order to aid optical

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differentiation of tissue components. Until the middle 19th century basically all dyes were natural products extracted in most cases from plants and animals. The dyes derived from plant e.g haematoxylin from Mexican tree (*Haematoxylon campechianum*), the dye derived from insect e.g carmine from body of a female insect (Dactilopus cacti); orcein dye was derived from Crocus sativus [5].

The genus Erythroxylum includes approximately 230 species distributed across the tropics [2]. The tree is widely distributed throughout the tropical Americas from southern Mexico to a far south and Paraguay [6]. Redheart's vibrant color quickly fades to a reddish brown in direct sunlight, but fortunately, there are some well-known and relatively simple means by which color change in exotic lumber can be prevented or slowed [6].

Studies with Erythroxylum species led to the isolation of secondary metabolities such as flavonoids, alkaloids, tannins, terpenes and phenylpropanoids that exhibit anti-oxidant activity, anti-carcinogenic, anti-inflammatory activity among others to be operated with pharmaceutical purposes [7].

The wood of Erythroxylum has been used for fence post and poles, flooring and sometimes for local house building, bridges, boat building and tools handles [8]. It has also been observed that ladies of Ebonyi state use it mostly for cosmetic reasons especially during its new yam festival where ladies use it to paint and look attractive to their male counterpart.

The quest for an alternative natural dye is due to scarcity of most commonly used nuclei and cytoplasmic stains (such as haemotaxylin and eosin) and special stain in histology coupled with their high cost. Hence the stimulation of this research that seeks to provide a better, eco-friendly organic and bio-degradation histological dye. This study therefore elucidate ethanol and ethylacetate wood extract of erythroxylum (red heart) staining reaction as a novel modification of Periodic acid Schiff, Haematoxylin Van Gieson and popular haematoxylin eosin stains on kidney, liver, lung and intestinal section of Rattus norvegicus respectively.

Materials and Method

Study Design

This is experimental research designed, log of Erythroxylum wood was gotten from timber shed, Sawed to dust in Abakaliki Timber Shed Ebonyi State and further dried and ground into powder using grinding machine. The wood powder extraction was done at the Histopathology Unit Department of Medical Laboratory Science, Ebonyi State University Abakaliki.

Materials Used for the Study

Rattus norvegicus, slides, cover slips, Ehrlich's haematoxylin, Harris haematology, grades of alcohol, 1% acid alcohol, Scott's tap water, xylene, 1% eosin, mountant, microscope, Erythroxylum plant wood, absolute alcohol, 70% alcohol, acetone, water Harris haematoxylin, Van Gieson Stain, Whatman No 1 filter paper, ethyl acetate, water, potassium alum, acetic acid, periodic acid, Schiff reagent, Erythroxylum wood powder and litmus paper.

Extraction of Erythroxylum Wood with 100% Alcohol and Ethyl Acetate

Extraction was carried out based on methods previously described by [10]. The Erythroxylum wood powder was further ground using an electric grinding machine 80g of the powder was dissolved in 400ml each of 100% ethanol and ethyl acetate and soaked for 72 hours. The solutions were filtered using Whatman No.1 filter paper. The filtrates were allowed to evaporate after exposing them in a flat bottom beaker and 0.4g of the powder was obtained from the evaporated ethanol solution and 0.38g from ethyl acetate solution, this was done severally to obtained the required quantity for the analysis. The percentage yield of the powder was calculated as:
Percentage yield = weight of the dry extract X 100/weight of dry powdered erythroxylum

Stain Preparation

3g of the both wood extracts were dissolved in 70% alcohol to give a 3% staining solutions. The PH of stain was 3.0 when tested using a litmus paper.

Slide Preparation and Staining

Three serial sections were obtained from a block of a histological processed lung, liver and Kidney tissue sections of Rattus norvegicus and labelled A, B and C. Section As served as controls, and were treated with routine Harris Haematoxylin and eosin, Weigert's Iron Haematoxylin and Van gieson solution and Periodic Acid Schiff solution respectively. Section Bs'and Cs' were stained and treated with the primary stains as in group A sections' and subsequently stained and modified with the prepared solutions of ethanol and ethylacetate extract of erythroxylum respectively.

Staining Procedure for Haematoxylin and Eosin Method (Control)

The sections were dewaxed in three changes of xylene 5 minutes each, hydrated in descending grades of alcohol 5 minutes each and washed with water. It was stained in Harris Haematoxylin solution for 20 minutes and washed with water. It were differentiated

in 1 % HCL in 70% alcohol for a dip and then rinsed in water. Blued in running tap water for 10 minutes and were counter stained with 1% alcoholic eosin for 2 minutes. It was dehydrated with ascending grades of alcohol cleared with xylene and mounted with DPX [9]

Weigert's Iron Haematoxylin Van gieson (Control)

The sections were dewaxed in three changes of xylene 5 minutes each, hydrated in descending grades of alcohol 5 minutes each and were all washed with water. The nuclei were all stained with Weigert's Iron Haematoxlin solution for 10 minutes and then rinsed in running tap water for 10 minutes and subsequently counter stained with Van gieson solution (mixture of 100ml Saturated picric acid solutions and 10ml 1% acid fuchsin solution) for 5 minutes. Further rinsed in distilled water was done and dehydration, cleared and mounted in DPX following standard procedure by Okorie, 2021.

Periodic Acid Schiff Technique (control)

Sections were deparaffinized in three changes of xylene 5 minutes each, hydrated in descending grades of alcohol 5 minutes each and all washed with water. Oxidized in periodic acid for 10 minutes and thoroughly washed in tap water, stained in Schiff's reagents for 20 minutes and rinsed in distilled water. Counter stained progressively in Harris Haematoxylin and blued in tap water, dehydrated, cleared and mounted in DPX mountant [9].

Modification of Haematoxylin and Eosin Method: Haematoxylin Ethanol and Ethylacetate Erythroxylum Extracts Staining procedure

The sections (Band C) were dewaxed in three changes of xylene 5 minutes each, hydrated in descending grades of alcohol 5 minutes each and washed with water. It was stained in Harris Haematoxylin solution for 20 minutes and washed with water. It were differentiated in 1 % HCL in 70% alcohol for a dip and then rinsed in water. Blued in running tap water for 10 minutes and sections (B and C) counter stained with 3% ethanol and ethylacetate erythroxylum extract for 2 minutes respectively and rinsed in distilled water, dehydrated, cleared and mounted in DPX.

Modification of Weigert Iron Haematoxylin Van gieson: Weigert Iron Haematoxylin Ethanol and Ethylacetate Erythroxylum Extracts Staining procedure

The sections (B and C) were dewaxed in three changes of xylene 5 minutes each, hydrated in descending grades of alcohol 5 minutes each and were all washed with water. The nuclei were all stained with Weigert's Iron Haematoxlin solution for 10 minutes and then rinsed in running tap water for 10 minutes and subsequently counter stained Section B with 3% ethanol erythroxylum extract and section C with 3% ethylacetate erythroxylum extract for 5 minutes and rinsed in distilled water dehydrated, cleared and mounted in DPX.

Modification of Periodic Acid Schiff's Technique: Periodic Acid Ethanol and Ethylacetate Erythroxylum Extracts Staining procedure

Sections (B and C) were deparaffinized in three changes of xylene 5 minutes each, hydrated in descending grades of alcohol 5 minutes each and all washed with water. Oxidized in periodic acid for 10 minutes and both sections stained with 3% ethanol and ethylacetate erythroxylum extract for 20 minutes respectively and washed thoroughly in running water, counter stained progressively in Harris Haematoxylin and blued in tap water for 10 minutes , dehydrated in ascending grades of alcohols (70%, 90%,Absolute I, II and III). Cleared and mounted in DPX mountant

Statistical Analysis

Graphpad Prism 9 software was employed for data analysis. Statistical analysis was carried out between treated groups and control using student t-test. $P \leq 0.05$ was considered statistically significant.

Results

The results of phytochemical composition of ethanol and ethyl-acetate extracts of Erythroxylum shown that ethanol extracted higher levels of these phytochemicals (flavonoids = tannins > terpeniods > glycosides > alkaloids > steroids > saponins > HCN) than ethyl acetate except flavonoids that shown higher in ethyl acetate extract (Figures 4.1a-b).

Table 4.1: Phytochemical composition of *Erythroxylum* wood Ethanol Extract

STAIN SOLUTION	TIRI ETHANOL	TIR2 ETHANOL	TIR3 ETHANOL
TANNIN mg/100g	*1088.452*	*1084.357*	*1089.271*
FLAVONOID Mg/100g	*1079.032*	*1074.194*	*1070.968*
HCN Mg/g	*0.252869*	*0.260433*	*0.266917*
SAPONIN Mg/g	*0.393275*	*0.384255*	*0.398687*
STEROID Mg/g	*0.758597*	*0.775455*	*0.786694*
GLYCOSIDES Mg/g	*542.7632*	*570.7237*	*585.5263*
ALKALOID Mg/100g	*511.6641*	*538.8802*	*534.2146*
TERPENOID Mg/100g	*797.1698*	*787.7358*	*799.0566*

Table 4.2: Phytochemical composition of *Erythroxylum* wood Ethylacetate Extract

STAIN SOLUTION	T2RI ETHYLACETATE	T2R2 ETHYLACETATE	T2R3 ETHYLACETATE
TANNIN mg/100g	*907.4529*	*911.5479*	*917.2809*
FLAVONOID Mg/100g	*1227.419*	*1239.516*	*1231.452*
HCN Mg/g	*0.124273*	*0.131837*	*0.13616*
SAPONIN Mg/g	*0.30127*	*0.312094*	*0.321114*
STEROID Mg/g	*0.247247*	*0.264104*	*0.241627*
GLYCOSIDES Mg/g	*330.5921*	*325.6579*	*319.0789*
ALKALOID Mg/100g	*345.2566*	*339.8134*	*364.6967*
TERPENOID Mg/100g	*250.9434*	*249.0566*	*256.6038*

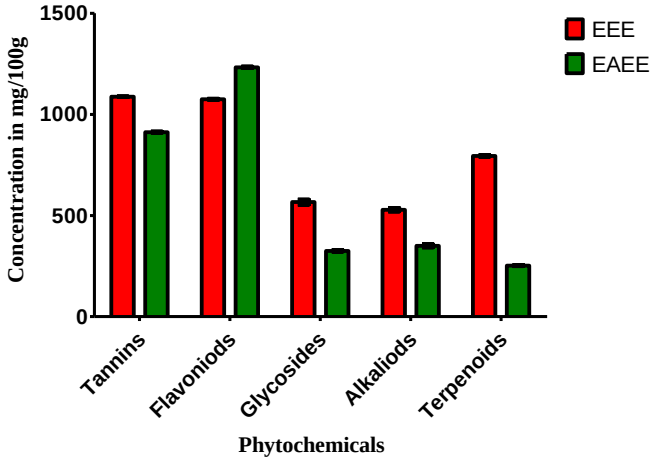

Figure 4.1a: Phytochemical Composition in Ethanol and Ethyl-acetate Extracts of *Erythroxylum*. Data are shown as mean ±S.D (n=3). EEE(Ethanol extract of Erythroxylum) and EAEE (Ethyl acetate extract of Erythroxylum).

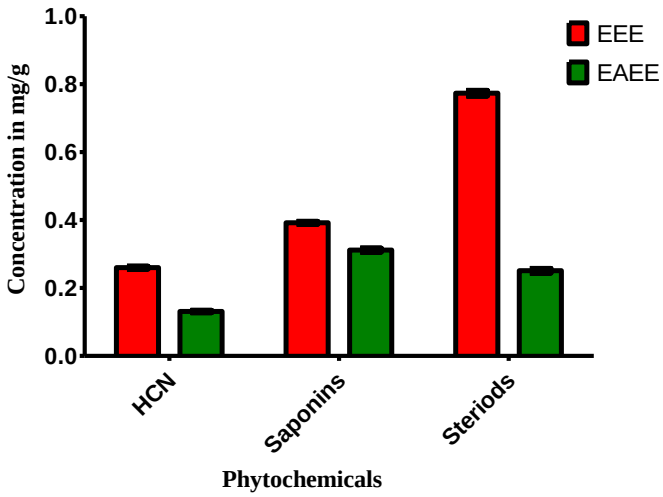

Figure4.1b: Phytochemical Composition in Ethanol and Ethylacetate Extracts of *Erythroxylum*. Data are shown as mean ± S.D (n=3). EEE(Ethanol extract of Erythroxylum) and EAEE(Ethyl acetate extract of Erythroxylum).

Photomicrographs of stained sections

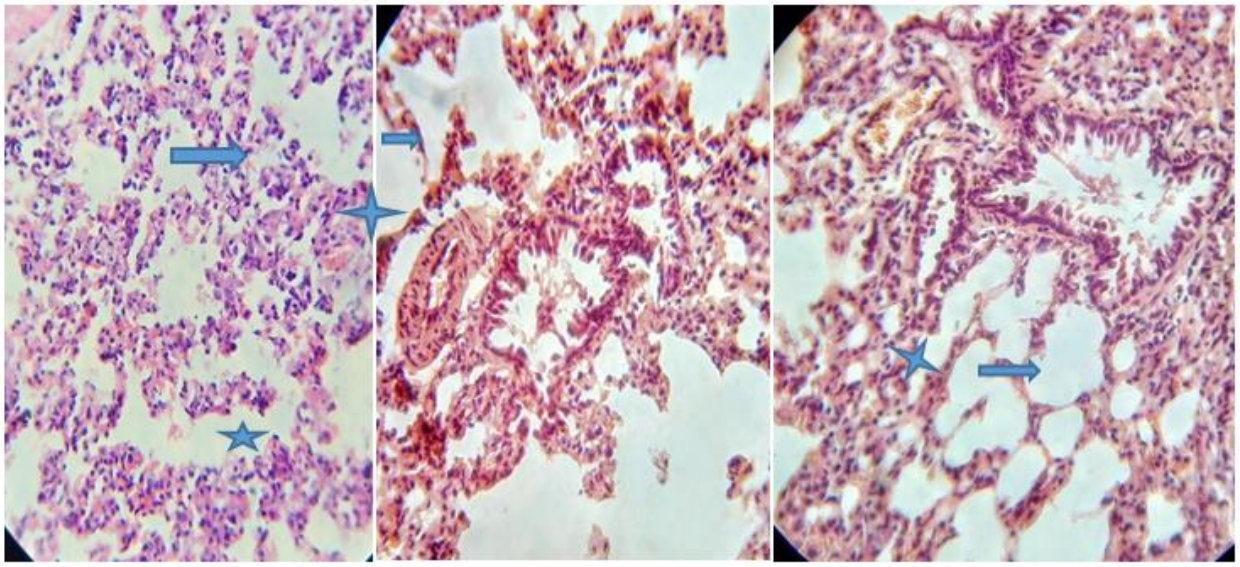

Figure 4.2a Mag. X40 **4.2b Mag. X40** **4.2c Mag. X40**

Figure 4.2a is a lung section of Rattus norvegicus stained with haematoxylin Eosin control; the sections shown a good demonstration of air spaces (blue arrow) and alveoli septa (blue star). 4.2b is a lung tissue stained with Haematoxylin ethanol extract of erythroxylum; the staining reaction demonstrated comparable histology details with fig. 4.2a and similar details were also seen in figure 4.2c which was stained with haematoxylin ethyl acetate extract of erythroxylum having the alveolar space(blue arrow) , alveolar septa(blue star) well demonstrated.

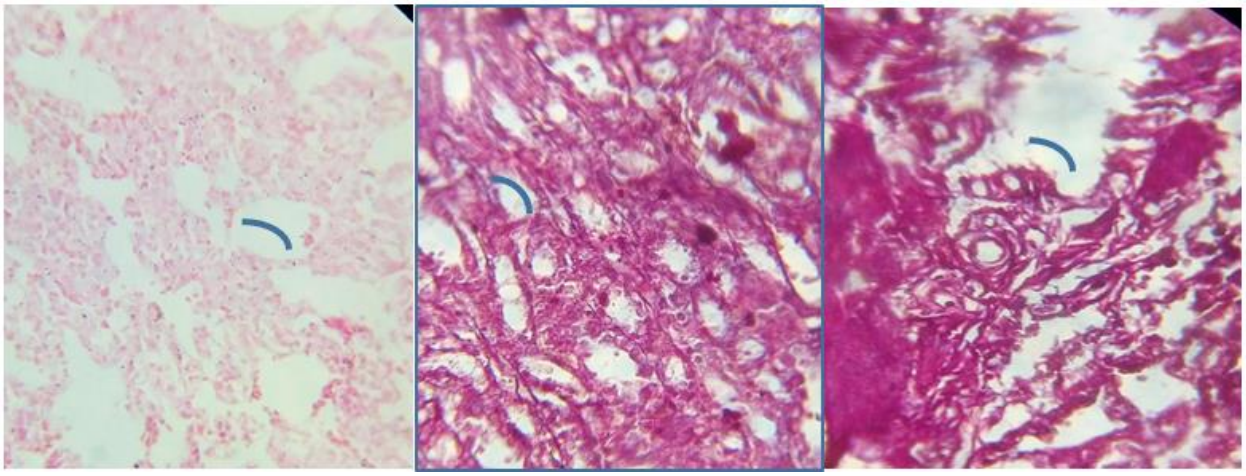

Figure 4.3a Mg.X40 4.3b X40 4.3cX40

Figure 4.3a is a lung tissues stained with Weigert Iron haematoxylin Van gieson stain, the section selectively stained the pneumocytes and had good demonstration of the air space. Same comparable staining reaction were seen in a deeply stained lung sections fig. 4.3b and 4.3c respectively. See the blue arcs 4.3b is 3% ethanol erythroxylum extract counter stained lung section and 4.3c ethy acetate erythoxylum counter stained lung section

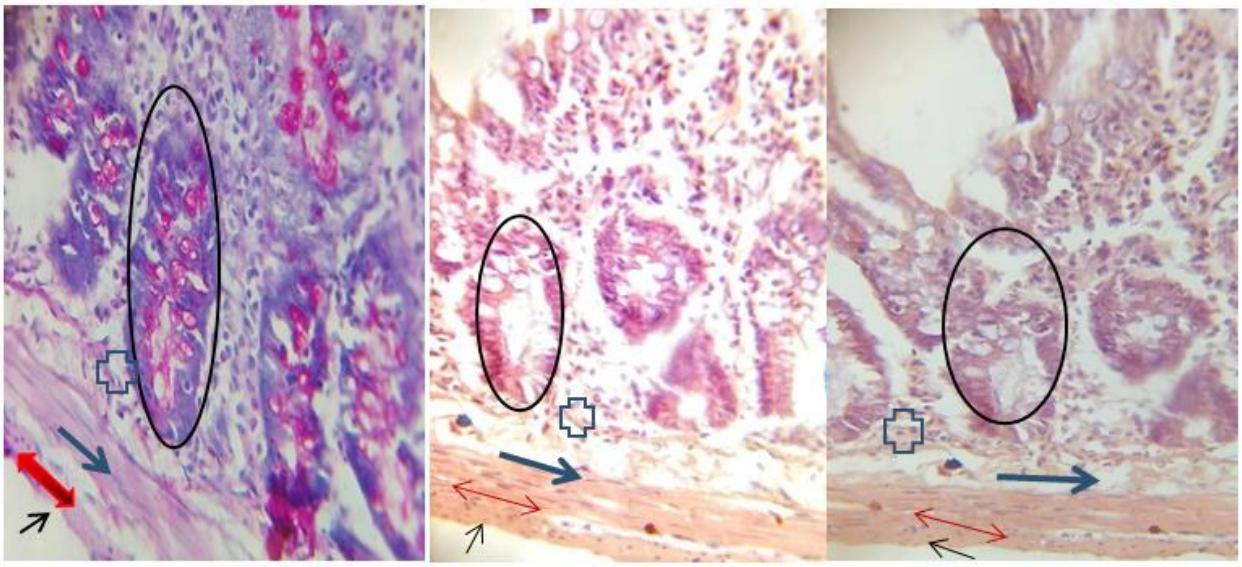

Figure 4.4a Mag. X40 Figure 4.b Mag X40 Figure 4.c Mag. X40

Figure 4.4a is an intestinal section of Rattus norvegicus stained with Periodic acid Schiff; the section shown good demonstration of intestinal layers. Serosa (black arrow), muscularis (double head red arrow), sub mucusa (blue arrow), Mucosa (blue cross) and the gastric gland (oval). The above description of periodic acid staining reaction of the intestine tissue is greatly comparable to Figure 4.4b (Periodic acid ethanol erythroxylum extract and) 4.4c Periodic acid ethyl acetate erythroxylum extract).

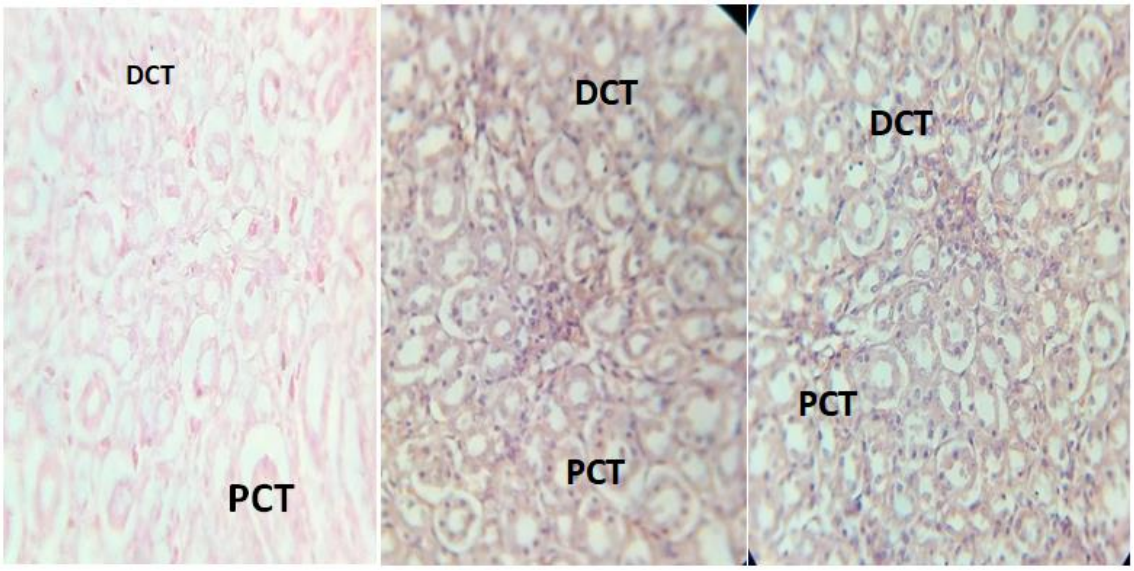

Figure 4.5a X40 Figure 4.5b Mag. X40 Figure 4.5c Mag.X40

Figure 4.5a is a kidney section of Rattus norvegicus stained with Weigert haematoxylin Van Gieson solution; the section shown good demonstration of podocytes, distal convoluted tubules (DCT) and proximal convoluted Tubules (PCT). Figure 4.5b and 4.5c are kidney sections of Rattus norvegicus stained with Weigert haematoxylin ethanol erythroxylum extract and ethylacetate erythroxylum extract solution respectively. The section shown a comparable and better optical differentiation of the tubular structures and podocytes

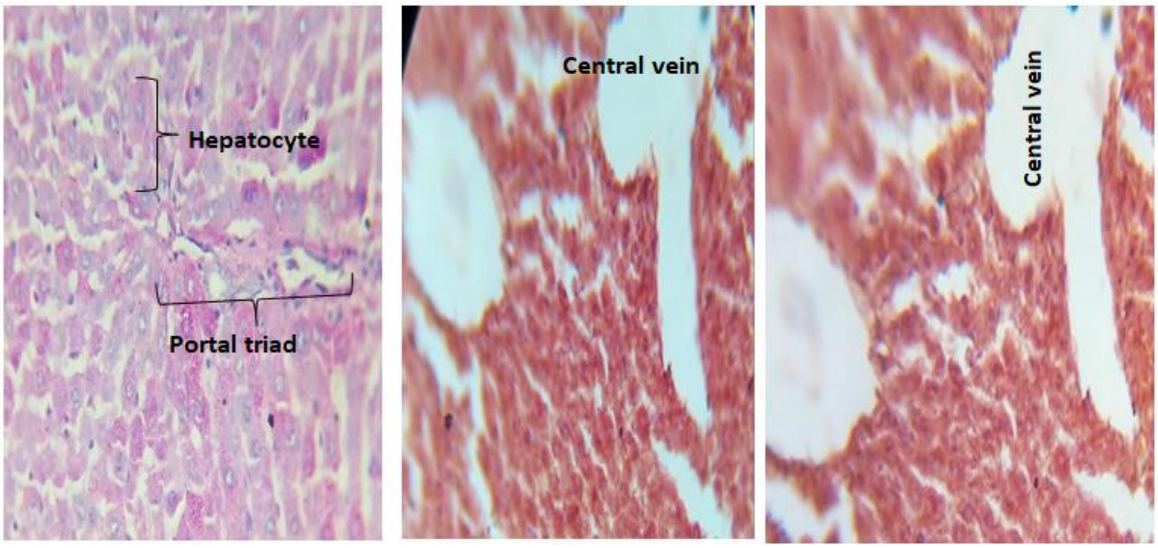

Figure 4.6a Mag. X40 Figure 4.6b Mag. X40 Figure 4.6c Mag.X40

Figure 4.6a is liver section of Rattus norvegicus stained with periodic acid Schiff solution; the stain revealed hepatocytes, portal triad and sinusoidal spaces among other histology details. Figure 4.6b and 4.6c are liver sections treated with Periodic acid and counter stained with ethanol erythroxylum extract and ethylacetate erythroxylum extract solution respectively. The section revealed a comparable hepatic details as seen in periodic acid schiff solution.

Discussion

In this study, Erythroxylum (erythroxylon) also known as redheart wood, is a genus of tropical flowering plants in the family Erythroxylacae [1]. Wild species of Erythroxylum are being described and subjected to new studies identifying their potential for future development of medicines [4]. In histopathology, stains are substances or biological dyes, which colour tissues in order to aid optical differentiation of tissue components. Until the middle 19th century basically all dyes were natural products extracted in most cases from plants and animals.

Elements such as nuclei would have high affinity for basic stain while cytoplasm, which is basic in character would have an affinity for acid stain. However, there are other natural and synthetic dyes which does not require addition of acid and base .An example of such a stain is commonly used as a counter stain for hematoxylin**.** By performing these experiments, we examined a lung section of Rattus norvegicus stained with haematoxylin Eosin control; the sections shown a good demonstration of air spaces and alveoli septa, in this study we were able to establish that a similar lung tissue stained with Haematoxylin ethananol extract of erythroxylum demonstrated a comparable histology details as haematoxylin Eosin control, similar details were also shown when stained with haematoxylin ethyl acetate extract of erythroxylum having the alveolar space , alveolar septa well demonstrated**.**

This study also demonstrated a kidney section of Rattus norvegicus stained with Weigert haematoxylin and counter stained with Van Gieson solution; the section shown a good demonstration of podocytes, distal convoluted tubules (DCT) and proximal convoluted Tubules (PCT),similarly kidney sections of Rattus norvegicus stained with Weigert haematoxylin and counter stained with ethanol erythroxylum extract and ethyl acetate erythroxylum extract solution respectively, shown a comparable and better optical differentiation of the tubular structures and podocytes.

Lastly we demonstrated a liver section of Rattus norvegicus stained with periodic acid Schiff solution; the stain revealed hepatocytes, portal triad and sinusoidal spaces among other histology details. A similar liver sections treated with Periodic acid and counter stained with ethanol erythroxylum extract and ethyl acetate erythroxylum extract solution respectively, revealed a comparable hepatic details as seen in periodic acid schiff solution stained section. This research work has shown a comprehensive comparison between routine histological stains and ethanol erythroxylum extract and ethylacetate erythroxylum extract solution on tissue sections on the basis of novel modifications

From this study, the extract has shown to have a similar effect and result as compared to other histological stains on tissue section, it also demonstrated a better optical differentiation when compared to Van Gieson solution. It can be hypothesized that the phytochemical composition in Ethanol and Ethyl-acetate Extracts of Erythroxylum directly has an effect in the better optical differentiation between tissue sections. It is therefore of importance that future studies of Erythroxylum should be encouraged.

Conclusion

Erythroxylum is a promising histological stain which is natural and readily available. The dye may serve in the demonstration of tissue constituents and may also serve as a useful cytoplasmic stain to replace other dyes for histological diagnosis of diseases and demonstration of the normal histological architecture of tissues and most importantly it aids comparability with the control as a modification of Schiff solution in Periodic Acid Schiff. Therefore, further studies should be carried out to recognize the accurate nature and components of the dye extracted in ethyl alcohol. The recognition of dynamic ingredients of the dye will open a new way of research in the field of dye. The dye extracted in ethyl alcohol should also be tested for bacteria and fungi as a histological stain.

Conflict of Interest

Authors declare no conflict of interest

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