International Journal of Research in Medical Sciences
Adisa JO et al. Int J Res Med Sci. 2017 Mar;5(3):1058-1065
www.msjonline.org
pISSN 2320-6071 | eISSN 2320-6012

DOI: http://dx.doi.org/10.18203/2320-6012.ijrms20170662

Original Research Article

A study of various modifications of *Lawsonia inermis* (Henna) leaf extract as a cytoplasmic stain in liver biopsies

James Olayiwola Adisa[1], Khadijah Khalid Musa[1], Ejike Chukwudi Egbujo[2]*, Ijeoma Matinda Uwaeme[1]

[1]Department of Medical Laboratory Science, University of Jos, Plateau State Nigeria
[2]Meena Histopathology and Cytology Laboratory, Jos Plateau State Nigeria

Received: 05 January 2017
Revised: 13 January 2017
Accepted: 06 February 2017

***Correspondence:**
Ejike Chukwudi Egbujo,
E-mail: anatomyejike@yahoo.com

ABSTRACT

Background: The leaves of *Lawsonia inermis* (henna) is widely used as cosmetic agent for body art to colour the hair, skin and nails, and hence is considered as a candidate stain for tissues. *Lawsonia inermis* leaves contain a pigment which is the source of red-brown dye. The application of natural dyes for staining biological tissues will reduce cost and is more environmentally friendly.
Methods: The natural dye Lawson (2-hydroxy-1,4-napthaquinone) was extracted from the dry leave of Henna using two solvents (distilled water and ethanol). The two extracts were formulated into various staining solutions at different concentrations. The staining solutions were modified with potassium alum and phenol. Liver sections were stained using haematoxylin and eosin method alongside various modifications of the plant extract as counterstain at different timing and temperature conditions.
Results: The solutions were found to be acidic in nature and stained the cytoplasm golden brown with a well-defined cytoplasmic boundary of hepatocytes. The best staining effect was obtained with 1% concentration at room temperature for 10 minutes. This provides a measure of contrast between the nuclei, cytoplasm and connective tissue that is adequate for diagnosis. Phenol used as an accentuator has been found to improve the staining but caused swelling of cell. Mordanting with potassium alum did not cause swelling but did not give the kind of resolution as obtained with Phenol. Use of the henna extract at 60oC gave the poorest result which showed that high temperature adversely affect the staining reaction by presenting almost the same color to the structures irrespective of the solvent or concentration.
Conclusions: This findings suggest that Henna leave extract can be used as a suitable cytoplasmic connective tissue stain.

Keywords: Counterstain, Cytoplasmic stain, *Lawsonia inermis*, Natural dyes, Stain

INTRODUCTION

One important aspect of histopathology is staining of histological sections prior to microscopic examination. If sections of human tissue are examined under the microscope immediately after sectioning, they appear very dull, uninteresting and morphological differentiation is very difficult. To bring out the structure of the tissues clearly it has to be stained. In histology, there are two types of dyes, Natural dyes obtained from natural sources and Synthetic dyes produced through chemical reactions.[1] The commonest stain used in histopathology is the Haematoxylin and Eosin (H&E) stain, which colours the nuclei a dark blue or purple and stains the cytoplasm and connective tissue in shades of pink. Staining of various biological tissues by application of natural dyes will be

more cost effective and reduce their effects on human and environment.[2] It will also generate more employment for local farmers.

Henna is a naturally occurring plant, which grow wild in abandoned areas.[3] The leaves of the henna plant are the source of red-brown dye widely known as a cosmetic agent used to color hair, skin and nails and also for body art.[4]

The major pigment in henna leaf is lawsone (2-hydroxy-1,4-napthaquinone) $C_{10}H_6O_3$. This dyeing principle (*lawsone*) is contained in the dried leaves in concentration of 0.5 to 2 %. It is not present as a free molecule in the leaves, but is derived from it precursor, that is hennosides during henna preparation.[5] Phytochemical screening for Henna leaf sample reveals the presence of Naphthaqunone (hannotanic acid), Mannite, Tannic acid, Gallic acid, Crysophanic acid etc.[6]

Henna extract has been used for staining stem cross section of angiospermic plants like *Helianthus annuus*, and *Zea mays*.[7] It has been used as a natural counter stain in Gram's staining reaction.[8] Despite all these dyeing potentials of Henna extracts, it has not been reported to been use as histological stain especially as an alternative counter stain in H and E staining technique. This study therefore aims at employing the use of Henna leaves extract as a natural cytoplasmic stain in histopathology.

METHODS

The plant was obtained from a farm in Toro local government area of Bauchi state, Nigeria and identification of herbarium voucher specimen was carried out by a competent botanist as *Lawsonia inermis.*

Extraction of dye

The leaves of henna plant were dried in the shade, ground and sieved to powder for effective extraction. The dye was extracted using water and ethanol.

Aqueous extraction

Aqueous extraction was carried out using heat extraction method.[9] 200g of Henna leaves powder was weighed and soaked in 800 ml of distilled water, shaken and left for 24 hours. The solution was filtered using No1 Wattman filter paper. The filtrate was placed in a hot air oven at 40^{o}C for three days to get rid of water. The residue was scraped, ground, weighed and stored in a dried air tight container. The dried powder obtained was 37.2g giving a percentage yield of 18.6%.

Alcoholic extraction

Ethanol extraction was carried out using to the procedure described by Hafiz.[10] 400g of the powdered plant material was soaked in 1000ml of absolute ethanol, macerated three times and filtered. The filtrate was placed in a hot air oven for three weeks to completely get rid of water. The dried powder obtained is 32.25g giving percentage yield of 8.1%

After extraction of the henna dye, various modifications of the extract were prepared and used to stain sections of the liver as shown in Table 1.

Table 1: Systematic protocol of the various treatments and modifications of the extract used to stain the liver biopsy.

Systematic protocol of the various treatments and modifications of the extract used to stain the liver biopsy
Different concentrations (0.5g, 10g and 20g) of both aqueous and ethanolic extract were used at room temperature to stain for 10, 30 and 60minutes duration.
2.0 grams of aqueous and ethanolic extract were each treated with Potassium Alum(mordant) and stained at room temperature for a duration of 30minutes
2.0grams of aqueous and ethanolic extract were each treated with Phenol(accentuator) and stained at room temperature for a duration of 30min
Different concentrations (0.5g, 10g and 20g) of both aqueous and ethanolic extract were used at 25 ^{O}C, 37^{O}C and 60 ^{O}C to stain for a duration of 30min

Liver sections were obtained from archived paraffin wax blocks. Sections stained using the Haematoxylin and Eosin method was used as control alongside the candidate henna extracts.

All stained sections were mounted in DPX and examined under the microscope at x10 and x40 magnification. Photomicrographs of the sections were taken.

RESULTS

General observations

- In the extraction procedure, aqueous extract took 3 days to dry to powder but the ethanolic extract took three weeks to dry to powder.
- In the preparation of the staining solutions, the aqueous extract dissolved completely but the ethanolic extract leaves a residue.
- Addition of phenol to the staining solution of Henna extract leads to the formation of whitish particles.
- The hydrogen ion concentration of varying solutions of henna extracts were determine using pH meter and the results obtained are shown in Table 2. The result showed that the pure solutions of henna at normal conditions are acidic and the addition of potassium alum and phenol reduces the pH.

The control section of the liver stained with hematoxylin and eosin shows a clear differentiation with a pink-red

cytoplasm and a blue nuclear staining. The morphologic structures of the liver are visible. Cytoplasmic margins are however not as clearly demonstrated.

Table 2: Hydrogen ion concentration of various solutions of henna extracts.

Solution	pH value	Mean pH
0.5g aqueous extract dissolved in 100ml distilled water	4.25	4.19
1.0g aqueous extract dissolved in 100ml distilled water	4.18	
2.0g aqueous extract dissolved in 100ml distilled water	4.14	
0.5g ethanol extract dissolved in 100ml distilled water	3.43	3.10
1.0g ethanol extract dissolved in 100ml distilled water	3.12	
2.0g ethanol extract dissolved in 100ml distilled water	2.76	
100 ml aqueous extract solution with 2.5g of potassium alum	2.46	2.44
100 ml aqueous extract solution with 5g of potassium alum	2.45	
100 ml aqueous extract solution with 10g of potassium alum	2.40	
100 ml ethanol extract solution with 2.5g of potassium alum	2.22	2.21
100 ml ethanol extract solution with 5g of potassium alum	2.21	
100 ml ethanol extract solution with 10g of potassium alum	2.20	
100 ml aqueous extract solution with 1% of phenol	3.88	3.95
100 ml aqueous extract solution with 3% of phenol	3.95	
100 ml aqueous extract solution with 5% of phenol	4.01	
100 ml ethanol extract solution with 1% of phenol	2.66	2.64
100 ml ethanol extract solution with 3% of phenol	2.64	
100ml ethanol extract solution with 5% of phenol	2.63	

Photomicrographs in Figure 1 showed the cytoplasm of liver tissue stained greyish brown. The control (H and E) demonstrate the cytoplasm as pink-red. The connective tissue are stained golden brown and the nuclei are stained blue by the haematoxylin. Cytoplasmic boundries of hepatocytes are generally demonstrated by the three concentration of Henna but 1% concentration gave the best effect (Figure 1C and D). These observations applies to both aqueous and ethanolic extract (Figure 1A-F).

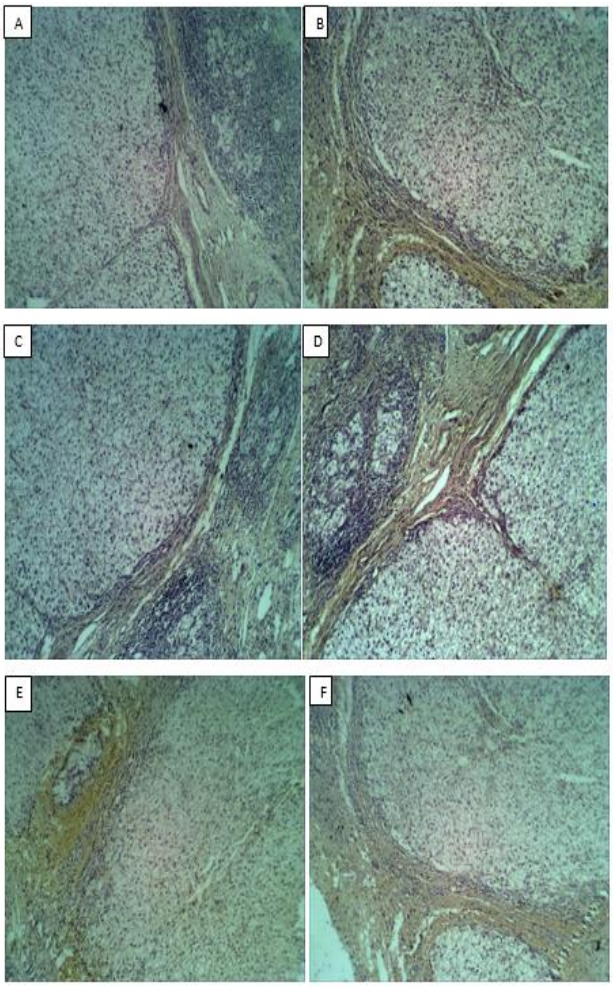

Figure 1: Photomicrographs showing treatment with various concentrations of henna extract; (A) Liver tissue section was treated with 0.5% aqueous extract of Henna for 30 minutes; (B) Liver tissue section was treated with 0.5% ethanolic extract of Henna for 30 minutes; (C) Liver tissue section was treated with 1% aqueous extract of Henna for 30 minutes; (D) Liver tissue section was treated with 1% of ethanolic extract of Henna for 30 minutes; (E) Liver tissue section was treated with 2% of aqueous extract of Henna for 30 minutes; (F) Liver tissue section was treated with 2% of ethanolic extract of Henna for 30 minutes.

Photomicrographs in Figure 2 showed that addition of potassium alum does not improve the staining reaction when compared with Figure 1, it stains the liver tissue but the outcome is not as good as that of pure henna without mordanting (Figure 2A-F).

Photomicrograph in Figure 3 shows that phenol appears to have a positive effect on staining with Henna especially with the ethanolic extract. Various concentrations of phenol were used (1%, 3% and 5%), but the 1% and 3% (Figure 3A-E) gave better resolution of structures. Addition of 5% phenol resulted in obliterating cellular details by excessive uptake of the henna dye (Figure 3F). It also seems that staining with

henna solutions treated with phenol, resulted in some swelling of the hepatocytes.

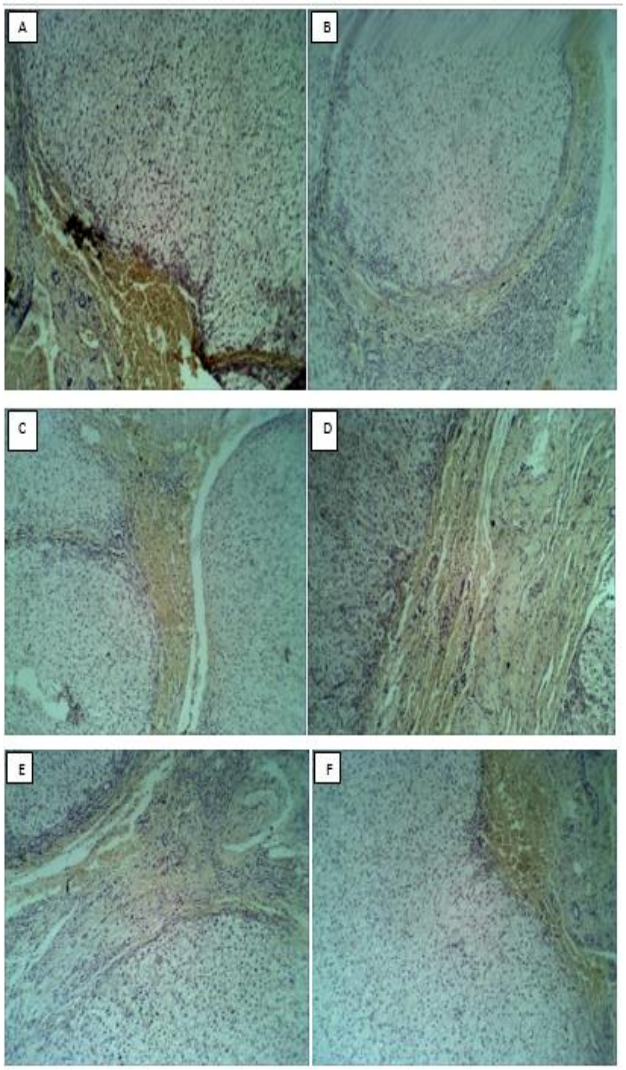

Figure 2: Photomicrographs Showing Treatment With Henna Extract And Various Concentrations of Pottasium Alum; (A) Liver tissue section was treated with 2.5g of potassium alum dissolved in 100ml of aqueous extract solution of Henna for 30 minutes (B) Liver tissue section was treated with 2.5g of potassium alum dissolved in 100ml of ethanolic extract solution of Henna for 30 minutes; (C) Liver tissue section was treated with 5g of potassium alum dissolved in 100ml of aqueous extract solution of Henna for 30 minutes; (D) Liver tissue section was treated with 5g of potassium alum dissolved in 100ml of ethanolic extract solution of Henna for 30 minutes (E) Liver tissue section was treated with 10g of potassium alum dissolved in 100ml of aqueous extract solution of Henna for 30 minutes; (F) Liver tissue section was treated with 10g of potassium alum dissolved in 100ml of ethanolic extract solution of Henna for 30 minutes.

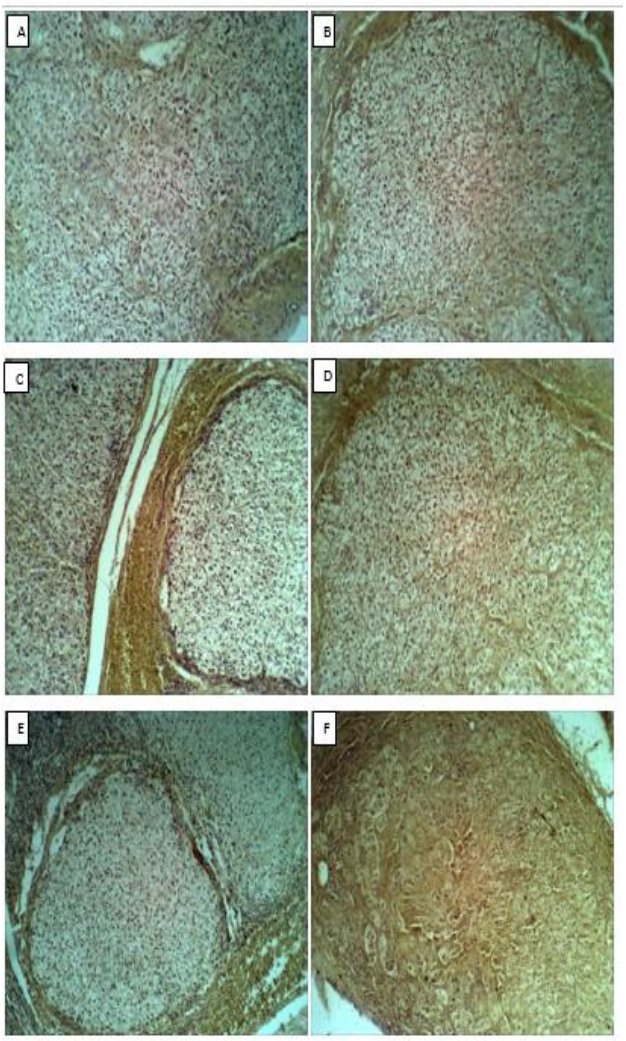

Figure 3: Photomicrographs showing treatment with henna extract and various concentrations of phenol; (A) Liver tissue section was treated with 1% phenol in 100ml of aqueous extract solution of Henna for 30 minutes; (B) Liver tissue section was treated with 1% phenol in 100ml of ethanolic extract solution of Henna for 30 minutes; (C) Liver tissue section was treated with 3% phenol in 100ml of aqueous extract solution of Henna for 30 minutes; (D) Liver tissue section was treated with 3% phenol in 100ml of ethanolic extract solution of Henna for 30 minutes; (E) Liver tissue section was treated with 5% phenol in 100ml of aqueous extract solution of Henna for 30 minutes; (F) Liver tissue section was treated with 5% phenol in 100ml of ethanol extract solution of Henna for 30 minutes.

Photomicrographs in Figure 4 shows that 37oC seems to favor 1% (Figure 4C and D). It's effect on 0.5% and 2% (Figure 4A, B, E and F) although not as good as 1% treatment provide a measure of contrast between the nuclei, cytoplasm and connective tissue. Unlike the control i.e., Hand E connective tissue of different types are not distinguishable.

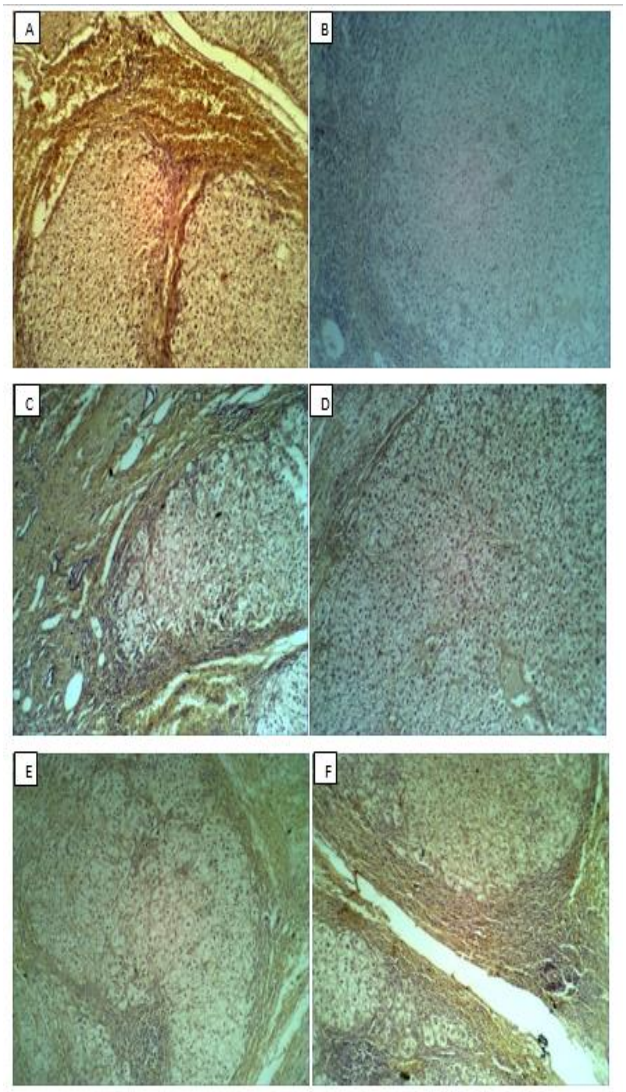

Figure 4: Photomicrographs showing treatment with various concentrations of henna extract at temperature of 37°c; (A) Liver tissue section was treated with 0.5g of aqueous extract of Henna for 30 minutes at 37°C; (B) Liver tissue section was treated with 0.5g of ethanolic extract of Henna for 30 minutes at 37°C; (C) Liver tissue section was treated with 1g of aqueous extract of Henna for 30 minutes at 37°C; (D) Liver tissue section was treated with 1g of ethanolic extract of Henna for 30 minutes at 37°C; (E) Liver tissue section was treated with 2g of aqueous extract of Henna 30 minutes at 37°C; (F) Liver tissue section was treated with 2g of ethanolic extract of Henna for 30 minutes at 37°C.

Photomicrographs in Figure 5 showed that temperature of 60°C adversely affect the stain. The Henna stain obliterated the nuclear stain(haematoxylin) thereby presenting all structures in the tissue with the same colour. This was the outcome irrespective of the solvent or concentration of the extract (Figure 5A–F).

Photomicrographs in Figure 6 shows that the result of staining with henna extract for 10 minutes gives an intense staining with clear differentiation as in Hand E. There is no interference between the primary stain and Henna (Figure 6A–F). Differenciation however decreases with increase in concentration (Figure 6A – E).

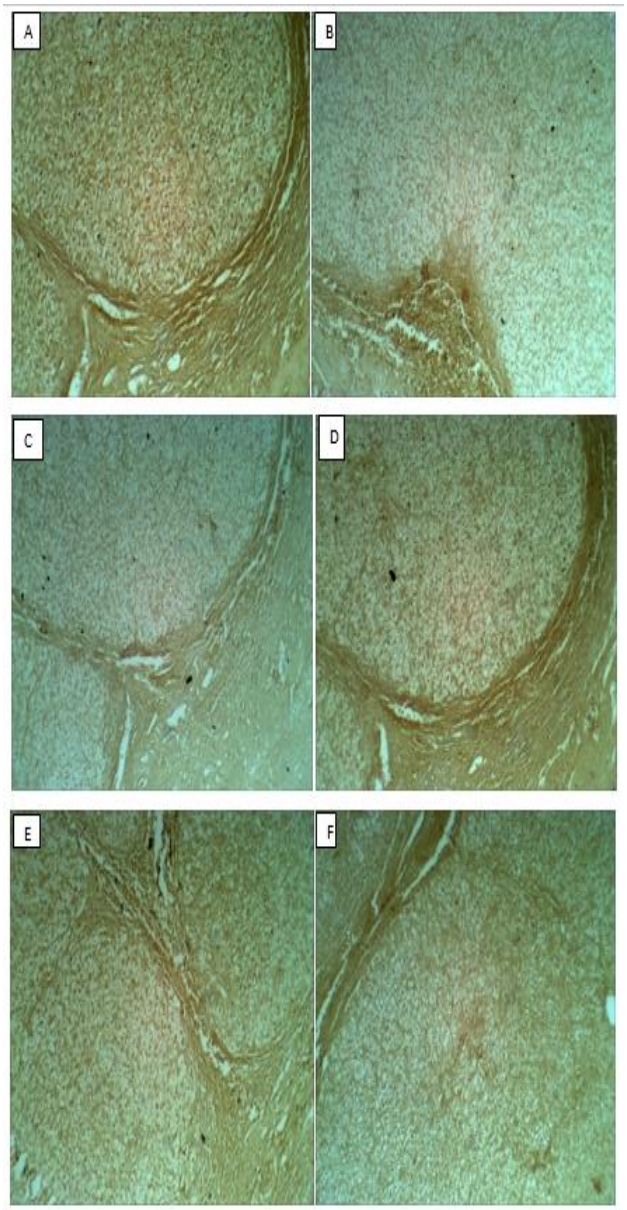

Figure 5: Photomicrographs showing treatment with various concentrations of henna extract at temperature of 60°c; (A) Liver tissue section was treated with 0.5g of aqueous extract of Henna for 30 minutes at 60°C; (B) Liver tissue section was treated with 0.5g of ethanolic extract of Henna for 30 minutes at 60°C; (C) Liver tissue section was treated with 1g of aqueous extract of Henna for 30 minutes at 60°C; (D) Liver tissue section was treated with 1g of ethanolic extract of Henna for 30 minutes at 60°C; (E) Liver tissue section was treated with 2g of aqueous extract of Henna for 30 minutes at 60°C; (F) Liver tissue section was treated with 2g of ethanolic extract of Henna for 30 minutes at 60°C.

Photomicrographs in Figure 7 showed that the liver tissue stain deeply giving a golden brown colour after 60 minutes of staining but differentiation of nuclei, cytoplasm and connective tissue was not enhanced (Figure 7A – F).

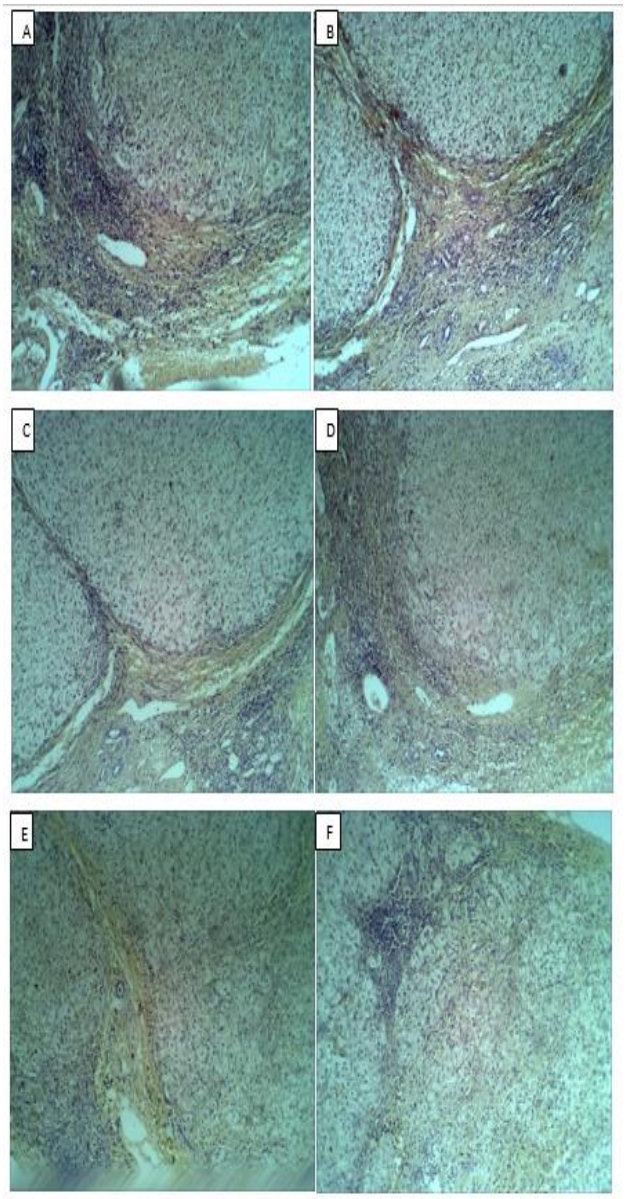

Figure 6: Photomicrographs showing treatment with various concentrations of henna extract for 10 minutes (A) Liver tissue section was treated with 0.5g of aqueous extract of Henna for 10 minutes; (B) Liver tissue section was treated with 0.5g of ethanolic extract of Henna for 10 minutes; (C) Liver tissue section was treated with 1g of aqueous extract of Henna for 10 minutes; (D) Liver tissue section was treated with 1g of ethanolic extract of Henna for 10 minutes; (E) Liver tissue section was treated with 2g of aqueous extract of Henna for 10 minutes; (F) Liver tissue section was treated with 2g of ethanolic extract of Henna for 10 minutes.

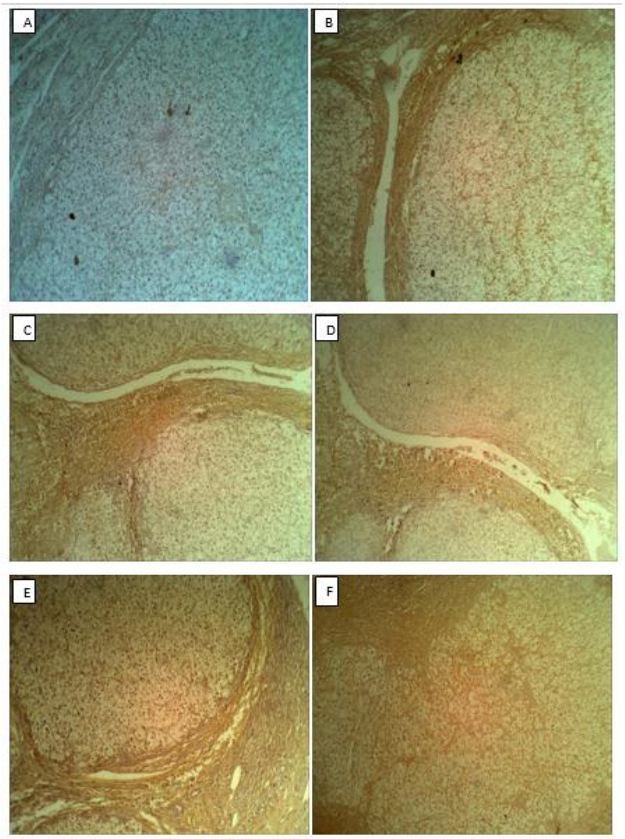

Figure 7: Photomicrographs showing treatment with various concentrations of henna extract for 60 minutes; (A) Liver tissue section was treated with 0.5g of aqueous extract of Henna for 60 minutes; (B) Liver tissue section was treated with 0.5g of ethanolic extract of Henna for 60 minutes; (C) Liver tissue section was treated with 1g of aqueous extract of Henna for 60 minutes (D) Liver tissue section was treated with 1g of ethanolic extract of Henna for 60 minutes; (E) Liver tissue section was treated with 2g of aqueous extract of Henna for 60 minutes; (F) Liver tissue section was treated with 2g of ethanolic extract of Henna for 60 minutes.

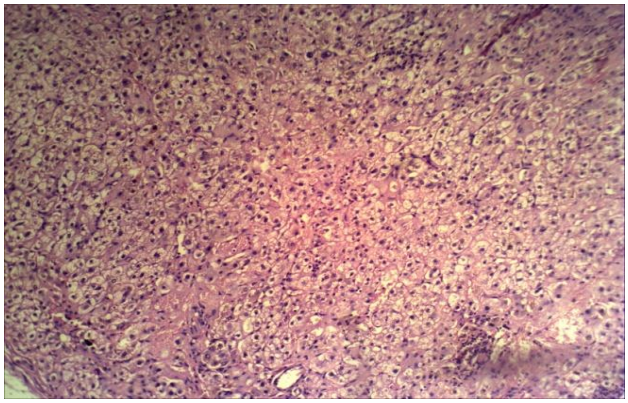

Figure 8: Photomicrograph showing control section stained with haematoxylin and eosin method.

DISCUSSION

General observations in the preparation of henna

In the extraction extraction of henna from the leaves, aqueous extraction yielded 18.6% while alcoholic extraction yielded 8.1% implying that water is a preferred solvent.

In the preparation of the working staining solutions, the aqueous extract dissolves completely but the ethanolic extract always leave a residue implying that within a given volume, saturation is attained earlier. Furthermore, this is an advantage in that it makes the use of henna cheaper.

Modifications of henna and effect on staining property

From the results, three different concentrations (0.5%, 1% and 2%) were used and both the aqueous and ethanolic extract stains the cytoplasm golden brown (Figure 1A-F) while in the control H and E, eosin stained the cytoplasm pink – red (Figure 8). Cytoplasmic boundries of hepatocytes are generally demonstrated by these three concentrations of Henna but 1% concentration gave the best effect (Figure 1C and D). 2.5%. 1.75% and 1% of Henna extract was used as a counter stain on *Lactobacillus spp* in gram staining without presenting any difference with their positive control.[10] The chemical component of Henna dye responsible for staining may be the Hydro (H^+) polar molecule. This also determines the pH of the solution. 1% Henna is known to have a pH of 4.18 and this makes it acidic and therefore its tendency to stain the cytoplasm.

The result of treatment with potassium alum (mordant) has no observable effect on the staining reaction with Henna extract (Figure 2A–F). It was observed that simple Henna solutions without mordanting stained the tissues better and the addition of a mordant does not improve its staining qualities. The use of mordant made the solution to become darker and the pH more acidic (3.65 to 2.32). The change in pH will certainly come with a change in ionic cloud and therefore the staining property. Potassium alum was used at different concentrations (2.5g, 5g and 10g) for both the aqueous and ethanolic extracts to improve the binding of the dye, i.e. mediate a dye–tissue interaction. The dye can only bind strongly to the tissue when the mordant acts as a link between the dye and the tissue. Our findings are similar to the report of Chukwu, where henna extract was mordanted with potassium alum to stain bacteria.[8] They also did not report improvement in quality of the stain. However, the various phytochemical constituents portray the henna plant extracts as a successful potential natural dye. For example in the context of staining, the red colour substance in the extract (tannin) makes the plant a true natural dye. Saponins are also known to reduce surface tension and this property enhances staining.[10]

Three different concentrations of phenol (an accentuator) (1%, 3% and 5%) were added to Henna staining solutions to see if it can improve the staining reaction of the stain. 1% and 3% appears to have a positive effect on staining with Henna especially with the ethanolic extract (Figure 3A–E). However, 5% phenol obliterated cellular details by excessive uptake of the henna dye (Figure 3F). This is attributed to the pH of the solution at that concentration that is found to be 3.30 which more acidic than with the simple solution. This implies that addition of phenol beyond 3% adversely affect the staining which is similar to the conclusion done by Hikmat, where extract of Henna was used effectively to stain plant tissues when employed in single staining without addition of an accelerator.[7]

Solutions of Henna extract were subjected to room temperature, 37^{o}C, and 60^{o}C to determine the temperature that best facilitate the staining reaction. From the result, treatment at room temperature gives a clear contrast between the structural component of the liver tissue (Figure 1-3 and 6) with both aqueous and ethanolic extract. 37^{o}C was found to favor 1% concentration of both aqueous and alcoholic extract (Figure 4C and D). The effect of 37^{o}C on 0.5% and 2% concentrations provide a measure of contrast between the nuclei, cytoplasm and connective tissue (Figure 4A, B, E and F) although not as good as 1% treatment. This applies to both the aqueous and ethanolic extract. At 60^{o}C, henna stain adversely affects the primary stain i.e heamatoxylin, thereby presenting the same colour to all structures in the tissue. It was observed that out of these temperature options, room temperature best facilitate the staining reaction since it gives the best contrast between the nuclei, cytoplasm and connective tissue of the liver tissue. This is similar to a study conducted by Chukwu, where Henna extract was used to stain both gram positive and gram negative bacteria at room temperature.[8] The extract therefore has the potential of being employed as a counter stain in Gram's staining reaction as well.

Different concentrations of henna extract were applied for 10 minutes, 30 minutes and 60 minutes to determine the most suitable time that can best stain the liver tissue. From the results, staining for 10 minutes gives an intense staining with clear differentiation. There is no interference between the primary stain and Henna. Staining differentiations decreases with increase in concentration of the candidate stain (Figure 6A–E). Staining for 30 minutes also gave a clear differentiation with minimal interference with the primary stain (Fig.1, 2, 3, and 6). Staining for 60 minutes showed that the liver was stained deeply by Henna with minimal differentiation. This shows that prolong staining with Henna does not improve differentiation but rather increases interference with haematoxylin (Figure 7A–F). It was observed that staining for 10 minutes is the best duration to counterstain histological sections especially liver tissue using Henna extract. Eosin stained the control section for one minute. This is because eosin is more avid

to Henna extract. This is similar to the study conducted by Chukwu, where henna extract was used to counter stain bacteria isolates for 1 minute in Gram's staining.[8]

The control section of the liver stained with hematoxylin and eosin shows a clear differentiation with a bluish and pink-red coloration respectively (Figure 8). Staining with hematoxylin and Henna extract gives a bluish and golden brown color respectively (Figure 1 - 7). The morphologic structures of the liver are visible but there was more differentiation using Henna extract. The Cytoplasmic margins and the cell boundaries are more visible with the Henna extract than the conventional H and E method.

CONCLUSION

Aqueous or ethanolic extracts of henna is acidic and the use of 1% solution of the extract in water, applied for 10min on liver tissue, as a Cytoplasmic stain gave excellent result comparable to the conventional H and E method. It was further observed that mordanting, or use of an Accentuator or increased temperatures did not confer any additional advantage.

Funding: No funding sources
Conflict of interest: None declared
Ethical approval: The study was approved by the Institutional Ethics Committee

REFERENCES

1. Avwioro OG, Aloamaka PC, Ojianya NU, Oduola T, Ekpo EO. Extracts of Pterocarpus osun as a histological stain for collagen fibres. African J Biotechnology. 2005;4(5):460-2.
2. Avwioro OG. Histochemical Uses Of Haematoxylin - A Review Journal of Pharmacy and clinical Sciences. 2011;1.
3. Muhammad, Z, Mustapha, AM. Traditional Malay Medicinal Plants. Kuala Lumpar. Fajar Bakti Sdn Bhd. 1994.
4. Hanna R, Maciej JN, Lapinskey L, Adamowiz L. Molecular structure and infrared spectron of 2-hydrozyl 4-nephathequinone. Experimental marix isolation and theoretical hart tree – fock and post hart tree – flock study. Spec Act. 1998;54:1091-103.
5. Gallo FR, Multari G, Giambenedetti M, Federici E. Chemical fingerprinting of Lawsonia inermis using HPLC & HPTLC and densitomery. Phytochemical analysis. 2008;19(6):550-9.
6. Crombie L, Crombie WML, Whiting DA. (1990). Alkaloids of khat (Cathaedulis). Alkaloids. 1990;39:139-64.
7. Hikmat UJ, Zanbta KS, Ashfaq AK. Staining effect of dye extract from dry leaves of Lawsonia inermis linn(Henna) on Angiospermic stem tissue. Pakistan J Botany. 2011;43(1):383-9.
8. Chukwu OOC, Odu CE, Chukwu DI, Hafiz N, Chidozie VN, Onyimba IA. Application of Extracts of Henna (Lawsonia inamis) Leaves As a Counter Stain. African J Microbiology Research. 2011;5(21):3351- 6.
9. Tandon, S, Rane S. Decoction and HotContinuous Extraction Techniques. In: HandaS.S, Khanuja, S.P.S., Longo, G., and Rakhesh,D.D (eds) Extraction Technologies for Medicinal and Aromatic Plants, United Nations Industrial Development Organization and the InternationalCentre for Science and High Technology, Padriciano, Italy. 2002; 93-106.
10. Hafiz H, Chukwu OOC, Nura S. The potential of henna (lawsoniainermis) leaves extract as counter stain in gram staining reaction. Bayero J of Pure and Applied Sciences. 2012;5(2);56-60.

Cite this article as: Adisa JO, Musa KK, Egbujo EC, Uwaeme JM. A study of various modifications of Lawsonia inermis (Henna) leaf extract as a cytoplasmic stain in liver biopsies. Int J Res Med Sci 2017;5:1058-65.