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Lawsonia inermis Linn (Henna) Lyophilized Extracts as Alternate Stains May Deter Staining Efficacy in Histology Sections

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Abstract
Lawsonia inermis is a shrub widely cultivated as an ornamental and hedge plant while the leaves are used in many countries for hair dyeing, fingernails painting and eyebrows coloration. This study hopes to stop the monopolization of haematoxylin stain for histopathology investigations. Therefore, our study aimed at exploring the staining properties of L. inermis (Henna) freeze-dried extracts as an alternate stain. The leaves of L. inermis were dried and pulverized. After which, 2kg was macerated in 10L of cold and hot water respectively, and 7.5L of absolute ethanol for each extraction solvents; filtered and freeze-dried. The pH of the 3 paste-like freeze-dried extracts was determined using a pH meter. Kidney, intestine, tonsil, and lung tissues were cut at 3-5mm and processed using routine histological technique and sectioned at 3-5um with the rotary microtome. Different extracts were prepared by substituting haematoxylin powder in Gills and Weigert haematoxylin and eosin preparatory procedures. Modified versions were used to stain sections from lungs, tonsil, appendix, kidney, and intestine with varying staining procedures at altered timing respectively. Freeze-dried extracts of L. inermis poorly stained histology sections compared to conventional methods in this study while, iron mordant significantly enhanced staining uptake / affinity of L. inermis extract. Freeze-dried extracts of L. inermis is not effective for nuclear staining of tissue sections compared to conventional haematoxylin while alcoholic extract appeared to be a better option amongst the trio extraction methods.

Keywords: *L. inermis,* stains, freeze-drying, lyophilisation, haematoxylin.

INTRODUCTION
Lawsonia inermis (Henna) is a shrub, and small tree widely cultivated as an ornamental and hedge plant (Sharma *et al.,* 2016). It is commonly called henna in English, Laali in yoruba, Lalle in hausa and Nchanwu in igbo dialects (Olise *et al.,* 2018). It is widely cultivated commercially

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for the production of henna, which is a dye extracted from its leaves (Sharma, 1990; Oviedo *et al.,* 2012).

Oviedo *et al.* (2012) reported that *L. inermis* is a plant that grows wild in abandoned areas while, Sharma (1990) informed that the plant is widely cultivated in Middle Eastern and northern African countries respectively. The plant has been introduced widely throughout the tropics and sub-tropics used to enhance beauty, as a dyestuff and elsewhere as a commercial crop. *Lawsonia* leaves are used in many countries for hair dyeing, fingernails and eyebrows during religion festivals and marriages (Jørgensen *et al.,* 2014). It can also be used to dye leather, silk and wool (Sharma and Singh, 1992) and the major pigment in henna leaf is lawsone (2-hydroxy1,4-napthaquinone) C10H6O3 (Jørgensen *et al.,* 2014).

Fessenden and Fessenden (1998) informed that the presence of quinones in henna, gives it its dyeing properties. Adisa, *et al.,* (2017) have demonstrated that henna extract could be used in histopathological preparations as a naturally occurring stain and that henna extract contains a natural pigment called naphthoquinone. Adisa *et al.,* focused on the application of stains derived from henna extract on angiospermic stem tissues while suggesting it usage for histological staining, which is the core gap being tackled in this study. Also, the former dwelled majorly on plant tissues as against histology sections derived from human tissues in our study.

From the foregoing, there is scarcity of information on the use of *L. inermis* as an alternate stain for histopathology investigations. It is against this backdrop that the present study conceptualized the idea of exploring *L. inermis* extracts with the hope that the monopoly of haematoxylin dye will subside. After successful experimentation of this study, the histopathology laboratory stands to benefit, and will in turn create an alternative to haematoxylin stains. Therefore, the present study was to explore the staining properties of *L. inermis* in assessing histology sections.

MATERIALS AND METHODS

Plant Material and Identification

The leaves of *L. inermis* were obtained from Apana in Jattu village, Etsako west local government, Edo State, Nigeria. The plant was identified and authenticated at the Department of Plant Biology and Biotechnology, University of Benin, Benin City and was assigned a voucher number UBH-L368. Ethical approval for the study was obtained from the research/ethical clearance committee of Ministry of Health, Benin City with reference number HA.737/15

Preparation of Plant Extract

Lawsonia inermis leaves were washed with tap water, shade dried until devoid of moisture. The dried leaves were then grinded into fine powder and sieved. The powder was transferred to an airtight container with proper labelling. Cold aqueous, hot aqueous, ethanol extract were prepared as follow: The cold aqueous extract was prepared by macerating 2 kg L. *inermis* in 10 L of water in a bucket. This was left to stand for 24 hrs at room temperature with intermittent stirring while the extract was filtered using cheese cloth. The hot aqueous extract was prepared by macerating 2 kg of L. *inermis* in 10 L of boiling water, and left to stand for 24 hrs with intermittent stirring. The extract was filtered using a cheese cloth as well. For the ethanol extract, 2 kg of *L. inermis* leaf powder was macerated in 7.5 L of absolute ethanol. This was kept in a dark cupboard and left to stand for 72 hrs with intermittent stirring before being filtered using a cheese cloth. All filtrates were stored in the refrigerator before they were

lyophilized or freeze dried using a rotary evaporator to obtain the cold water, hot water and ethanol extracts respectively. The pH of the 3 paste-like L. inermis freeze-dried extract was determined using a pH meter. 1 g of the ethanol plant extract was dissolved in 10 mL of absolute alcohol, while 1g of the hot water and cold water extract were dissolved in 10mls of water.

Preparation of Sections

Kidney, intestine, tonsil, and lung tissues were cut at 3-5mm and processed according to standard histological procedures and sectioned at 3-5um with the rotary microtome.

Preparation of *L. inermis* Extract for Staining

Alum and iron hematoxylin were both prepared in order to ascertain the best mordant for the extract. The alum hematoxylin formula used was Gill's while iron hematoxylin was Weigert (Avwioro, 2010). The conventional method for the preparation of hematoxylin stains was used in preparing both alum and iron hematoxylins which served as controls. While in this experiment *L. inermis* extract was substituted in place of haematoxylin in the conventional staining method for Gills and Weigert staining methods respectively (Avwioro, 2010). The durations to which the histology sections were left in the staining bath containing the extracts were: 10 min, 20 min, 30 min, 45 min and 1 hr respectively.

Microscopy and Photomicrography

Histology stained slides were viewed with a light microscope by an experienced histopathologist while photomicrographs were obtained with a digital camera (Armscope MD900).

RESULTS

Ethanol and cold aqueous extracts had a pH of 6.5 respectively while, the hot aqueous extract had a pH of 6.9. Plate 1: A-H showed varying appearances of the stain uptake on various histological sections and at different timing. Preparation of *L. inermis* with weigert hematoxylin revealed the best staining result although poorly stained compared to the various timing (10 min, 20 min, 30 min, 45 min and 1 hr) and different freeze-dried extracts. Only ethanol extraction stained with Weigert H&E for 1hr showed a satisfactory stain uptake in the nucleus while that of ethanol (45 mins), stained next best compared to the control. In addition, all other extracts from varying durations stained poorly and most of which did not pick up stains and thus, apeeared that longer duration of staining is desirable in this study.

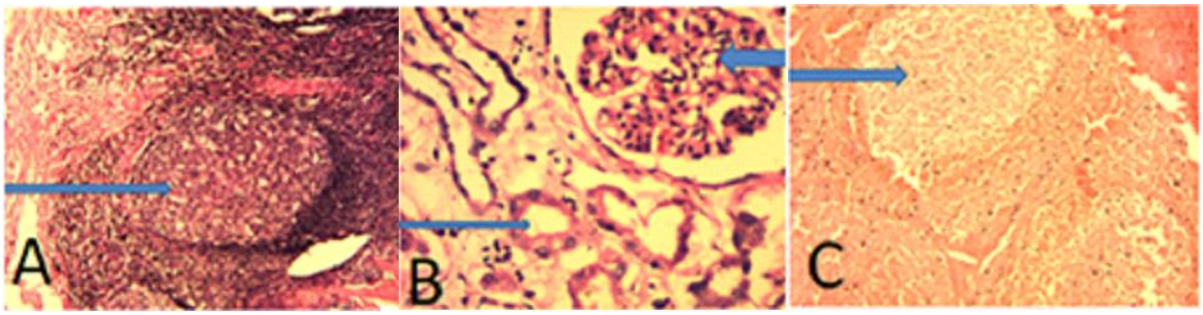

Plate 1: **A.** showed section of tonsil with lymphoid follicle (Arrow) stained with conventional Weigert's H&E and served as control. **B**. *L. inermis* cold extract in place of haematoxylin in Weigert's H&E staining of the kidney section for 1 hr revealed a near perfect staining uptake of the glomerulus (thick arrow) and tubules (thin arrow) and served as modified version. **C**. *L. inermis* (hot extract) in place of haematoxylin in Weigert's H&E staining of the tonsil section for 1 hr revealed a near perfect staining uptake of the lymphoid follicle (arrow).

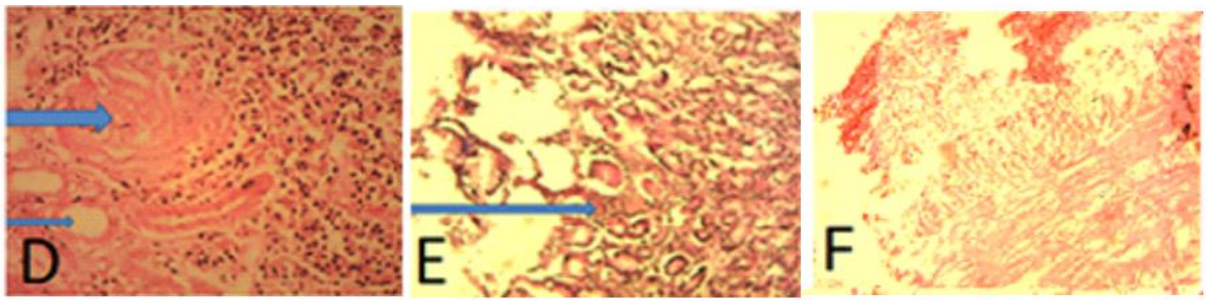

Plate 1: D. *L. inermis* (alcohol extract) in place of haematoxylin in Weigert's H&E modified version staining of the kidney section for 1 hr showed a near perfect staining uptake of the glomerulus (thick arrow) and normal tubules (thin arrow). **E**. Section of the intestine showed poor stain uptake of the epithelia layers (arrow) using Weigert's H&E (cold extract) for 45 mins. **F**. Section of the lungs showed poorly stained alveli space and bronchiole using the modified Weigert's H&E (hot extract) for 45mins.

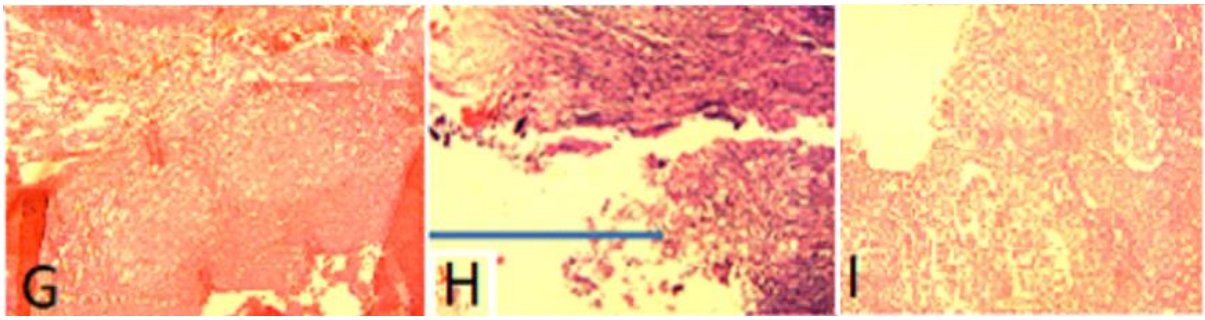

Plate 1: G. Section of the tonsil showed poor stain uptake of lymphoid follicle (arrow) with the modified Weigert's H&E (alcoholic extract) for 45 mins. **H**. Section of the appendix stained with the conventional Gill's H&E (control) erosion of surface epithelium (arrow). **I**. *L. inermis* cold extract in place of haematoxylin in Gill's H&E stained for 1 hr and served as modified version showed section of the intestine with near good stain uptake of the surface epithelium.

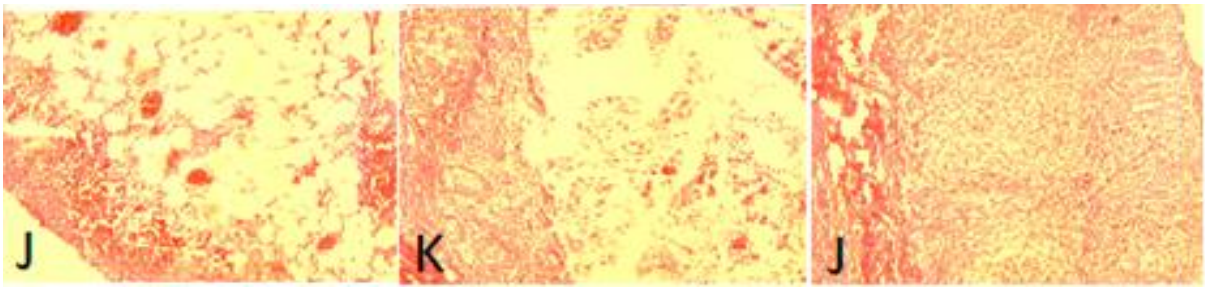

Plate 1: J. *L. inermis* cold extract in place of haematoxylin in Gill's H&E stained for 1 hr and served as modified version showed section of the intestine with near good stain uptake of the surface epithelium. **K**. *L. inermis* cold extract in place of haematoxylin in Gill's H&E stained for 1 hr and served as modified version showed section of the intestine with near good stain uptake of the surface epithelium. **L**. *L. inermis* cold extract in place of haematoxylin in Gill's H&E stained for 45 mins and served as modified version showed section of the intestine with near good stain uptake of the surface epithelium. All photomicrographs at x400 magnifications respectively.

DISCUSSION

Using an alum mordant for *L. inermis* extracts did not improve the staining quality in this study and may be an indication that an iron mordant is better off when using *L. inermis* as this has shown visible improvement on staining quality reported by Adisa, *et al.,* (2017). Recall that Adisa *et al.* (2017) earlier reported the treatment of *L. inermis* extract with potassium alum and observed no effects on staining uptake, which has been duly demonstrated in our study.

In comparison to the control stained with Wiegert histochemical technique for demonstrating

elastic fibre. It was observed that, only the cold aqueous extract stained for 1 hr gave a near satisfactory result despite that the staining uptake was poor. The hot and ethanol extracts gave patches of stains on the lung tissue sections, which were not defined. It thus means that differentiation from other components of the section was poor. On the same report, the nucleus did not pick up stains from the three extracts, which may be due to the use of the extract as a primary stain.

We observed that during the filtration process of the extract, especially the cold-water extract, our nails were stained for about 2 months before it finally faded off. After freeze drying, the extracts were used to stain slides with different staining procedures at different time intervals but it was observed that sections did not pick-up the stain properly. For further understanding, we used the freeze dried extracts to stain our nails overnight and the nails did not also pickup stain compared to our experience during the extraction processes. However, only the skin around the nails picked up the stains faintly. This observation strongly supports the report by Olise *et al.* (2018); wherein in *L. inermis* freeze-dried extract was reported to be relatively unfit for biological staining. They further stressed that though, the extract has staining capability, the reason for not picking up stains by biological specimens is poorly understood as at the time of writing their report though a purer form of the extract is recommended for the improvement of the staining ability. From our experience, we strongly suggest that vital staining components may have been lost while lyophilizing the extracts.

The present study is dissimilar to the work of Hafiz *et al.* (2015); in which *L. inermis* staining properties were investigated and found that the extract stained liver biopsies and gram staining respectively. The reason for the disparity may be due in part to the effects of freeze drying on staining ability of the extract. In support, the fact that sections did not pick up stains from the three different extraction methods may be suggestive of an impact of freeze-drying exerted on lawsone, which is the active staining component of the extract. On the other hand, Kunal *et al.* (2015) stated that freeze drying can cause loss of volatile compounds due to high vacuum. Adams (2007) earlier reported that various extracts have individual critical temperature, and anything below this temperature during freeze drying will lead to an instantaneous irreversible collapse of the phytochemical structure of the extracts leading to production of inferior quality of the extract.

This study suggests that poor staining uptake in this work may be attributed to the use of the extract as a basic dye instead of using it as an acidic dye. The decision was based on the pH of the various extracts, which was an average of pH 6.6. Though, this is not in tandem with Adisa *et al.* (2017) who reported that the pH of *L. inermis* aqueous and ethanol extracts used in their study were 4.2 and 3.1 respectively, and were used as acidic dyes. We therefore, suggest that the difference in pH may be as a result of denaturing or loss of lawsone or other phytochemical components of the extracts during freeze drying process, which may have gross impact on the staining ability of the extracts. However, irrespective of the different pH values obtained which was different from that of Adisa *et al.*, it is worthy of note that aqueous and ethanol extracts were used by the latter as acidic dyes while our study used a similar extract as a basic dye. Both extracts were aimed at staining different cellular structures i.e. cytoplasm and nucleus.

Another reason why our study did not pick up stain aside the freeze-drying indications may be because extracts were used as a dye instead of being used directly as a stain without freeze-drying. Hikmat *et al.* (2011); who used *L. inermis* ethanol crude extract as a direct stain without adding any further reagent and did not lyophilized the extract had perfectly stained sections.

Kannanmarikani *et al.* (2015) also reported that the crude extract of *L. inermis* has quality staining abilities though with a short shelf life. This may not have been the issue in this study as the present extracts were refrigerated for the entire duration of study and could have kept the dye stable.

The present extracts did not dissolve immediately it was introduced into their various solvents in the extraction phase of this experiment. They had to be stirred and mixed vigorously before extracts were completely dissolved which took about 10 minutes. This observation is vital for future experiment.

CONCLUSION

We suggest that freeze-dried extracts of *L. inermis* is not effective for nuclear staining of tissue sections compared to conventional haematoxylin while alcoholic extract appeared to be a better option amongst the trio extraction methods.

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