STAINING POTENTIALS OF LOCALLY SOURCED *CURCUMA LONGA, CNESTIS FERRUGINEA* AND *GARCINIA KOLA* ON LIVER AND KIDNEY TISSUES

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The use of eosin in histopathology may be restricted due to the discovery of its hazardous nature. Moreover, most developing countries can no longer afford the ever-increasing cost of the stains. Consequently, the need for evaluation of staining potentials of *Curcuma longa rhizome*, *Cnestis ferruginea* stem barks and *Garcinia kola* mesocap becomes paramount. In the present study, sohext extracts of the plant products were substituted for eosin (as counter stains) in the modified haematoxylin and eosin methods for the staining of kidney and liver tissue slides in order to study their staining potentials. Results showed that 100 mg/ml freshly prepared *C. longa* rhizome extracts was most effective in staining the slides; giving excellent cytoplasm staining. *C. ferruginea* extracts produced faintly stained pale red colouration of the cytoplasm. Tissue sections stained with *G. kola* pods extracts showed fairly good cyto-architectural distinctions. When each of the tissue slides was stained with only a single stain (mono staining) and 6-month old plant extracts, there was reduction in the intensities and clarity of features. The suitability of the plant products as counter stains as observed in the study could be attributable to their acidic nature which naturally stained the basic cytoplasm contents.

Key words: *Curcuma longa, Cnestis ferruginea, Garcinia cola,* ethanolic extract, aqueous extract.

INTRODUCTION

Stains are of great importance in histopathology. Tissues acquired from the bodies of human or animals are inconspicuous after tissue processing. Without tissue staining, the individual components of these tissues cannot be seen and appreciated. Hence, stains help in diagnosis of ailment because they allow easy identification of normal and pathological tissue structures by coloring the various components of the tissues. Stains are classified in various ways. They can be grouped as natural or synthetic (artificial); or according to their reactions, tissue affinities and major applications (Ochei and Kolhaktar, 2000). Also, they can be sub-divided into acid and base groups, a combination of which can produce a neutral stain.

Haematoxylin is extracted from the heartwood of a small tree, *Haematoxylin campechlanum* (Logwood), which originated in Mexico and has been cultivated in Jamaica. It is one of the most widely used dyes in histology which in its natural form has little or no staining capacity and requires oxidation to haematein, either naturally by contact with air or chemically with an oxidizing agent such as sodium iodate or mercuric oxide. Together with Eosin, haematoxylin forms Hematoxylin and Eosin staining method and is the most commonly used staining method in histopathology laboratories. The use of these dyes and other synthetic dyes is restricted due to the discovery of their hazardous nature, especially eosin. Also, most developing

countries can no longer afford the ever-increasing cost of these dyes. Consequently, the need for evaluation of staining potentials of indigenous plant products which are domestically available, easy to prepare and use as well as resistance to fading becomes paramount. Due to the essentiality and importance of staining in histopathology together with the hazards of using synthetic dyes and the cost of foreign dyes, it becomes essential to seek for natural, economic, easy to use, biodegradable, easily available, safe to both human and environmental health and more eco-friendly alternatives or supplements to the routine Haematoxylin and eosin stains, hence the importance of the present study.

C. longa is also known as 'Turmeric'. It is a rhizomatous herbaceous perennial plant of the ginger family (Zingiberaceae). It has a deep yellow-orange color (Avwioro et al., 2007). Extraction of the rhizome with ethyl alcohol, acetone or methylene chloride yields 6-10% oleoresin, (which contains 35-45% curcumin and its derivatives, demethoxycurcumin and bis-demethoxycurcumin, is collectively called curcuminoids). These compounds give turmeric its characteristic orange-yellow color (WHO, 1999). Previously, few researchers have experimented on the use of turmeric as a histological stain, regularly as a counter stain for cytoplasmic staining. These include: (a) *C. longa* as a histological dye to stain collagen fibres and red blood cells (Avwoiro et al., 2007); (b) *C. longa* as a histological dye to stain tissues extracted from the testis (Bassey et al., 2011). *C. ferruginea* is an erect or scan dent shrub or climber of deciduous forest and secondary shrub land widely dispersed in West Africa and other tropical parts of Africa. The phytochemical screening showed that the major active constituents of the aqueous root extract of *Cnestis ferruginea* are alkaloids, flavonoids, reducing sugars, phenols, saponins, steroids, anthraquinones, and glycosides. Anthraquinones is a source from which most synthetic dyes are derived. Glycosides contain sugar. Tannins play a role in protection from predators and could also serve as pesticide. Saponins are essential elements in the production of soaps (Trease and Evans, 2009). *G. kola* plant is popular in Southern Nigeria, usually found in tropical rainforest region. The fruits are reddish-yellow and each fruit contains 2-4 brown seeds embedded in orange-colored pulp (Iwu, 1993).

It could be observed from the foregoing that of all the plant products under study, only the staining potentials of *C. longa* has been moderately studied and documented. Besides, either aqueous or ethanolic extracts of the plant products have been slightly investigated. To the best of the authors' knowledge, there is only one published article on the use of both ethanolic and aqueous methods of extraction to study their staining potentials (Olise et al., 2018). Pathological features of tissues had not been previously highlighted nor the effects of mordant on the staining efficiency investigated. Determinations of their optimal staining concentrations are yet to be elucidated. Thus, the current study is aimed at investigating the staining potential of the aqueous and ethanolic, mordant and non-mordant extracts from the plant products on pathological and normal Liver and Kidney tissues. Kidney and liver were preferred because of their characteristic and easily noticed peculiar features.

MATERIALS AND METHODS

Materials and methodology employed in this study are in accordance with the standard operating procedures (SOPs) applied at the Histopathology Department of the Imo State University, Owerri. The chosen study area was the Histopathology Department of Imo State University, Owerri, Nigeria. Owerri is the capital city of Imo State in South Eastern Nigeria. Located in latitudes 5025II 50.23n and longitudes 702II 149.33E. It covers approximately 40 square miles (100 km^2). The climate of the area which enhances the growth and thriving of the plant products is tropical, with annual rainfall of about 1000 mm and temperature range between 22.7 and 33.7°C. Igbo is the predominant ethnicity in the area. The study population employed the use of some selected organs such as liver and kidney, obtained from some autopsy samples received from the Histopathology Department of the Federal Medical Centre Owerri. Plant samples were obtained from Nnewi and Owerri, Eastern Nigeria, branded at the herbarium of the Botany

Department of Imo state University and were given voucher numbers.

Sample size determination

Sample size determination was carried out using the method of Ari (2017) as follows:

ss= (Z2 x p (1-p)) / ME2

Where
ss = sample size
Z = the Z score for 95% confidence interval (as obtained from statistical table) which is 1.96
p = the standard deviation of the population (due to the fact that autopsy and biopsy samples population were unknown, the standard deviation of the population (P) was assumed to be 0.5)
ME = Margin of error which is 5% (5/100) = 0.05
Substituting, the sample size becomes:
1.962 x (0.5 (1-0.5)) / 0.052 = 385

Preparation of plant materials

The plant products were air-dried properly for 7 days. They were subjected to grinding with the aid of a mechanical grinder to powdery form, sieved and stored in a dry container. Aqueous and alcoholic extractions were then carried out on the powdered material, using sohext extraction method to acquire a pure extract which was used for staining.

Tissue processing

The tissues (3 mm thick) were processed using the rapid manual tissue processing method as follows: the organs were immediately fixed in 10% neutral buffered formalin for 48 h. After cutting them up with knives, they were dehydrated through the indicated grades and changes of ethanol for the stipulated period as follows: 70% ethanol (for 2 h), 90% ethanol I (for 2 h), absolute ethanol II (for I – 2 h), absolute ethanol III (for I – 2 h), and absolute ethanol IV (for I – 2 h). The tissues were cleared in two stages of Xylene (:xylene I for 2 h, xylene II for 2 h), placed in a mould containing molten paraffin wax. When the blocks were set, they were trimmed using a rotary microtome (SHANDON AS325) to expose the tissue surface and placed in an ice bath before sectioning at 5 microns. Sections were then floated onto a hot water bath set at 10°C below the melting point of paraffin wax. Albumenized slides were used to pick up the floating sections from the water bath. A hot plate was used afterwards to attach the sections firmly to the slides.

Staining procedure for fresh and 6 month old extracts:

The slide was dewaxed in xylene, stained in hematoxylin for 15 min, washed in tap water and differentiated in 1% acid alcohol. Blueing was done in running tap water for 5 min. The slide was stained with (a) *C. longa* (turmeric) solution for 10 min (b) *C. ferruginea* for 30 min (c) *G. kola* for 2 min; dehydrated in ascending grades of alcohol (50, 75, 95% and Absolute), cleared in xylene and mounted with DPX.

Staining procedure for mono-staining of slides

The slides were dewaxed in xylene and water. Slides were stained with: turmeric solution for 10 min (acidic ph); *C. ferruginea* for 30 min (acidic ph); *G. kola* for 2 min. The slides were washed in water, dehydrated in ascending grades of alcohol (50, 75, 95% and Absolute), cleared in xylene and mounted with DPX.

Intensity and clarity of staining with the plant products

Every stained slide was observed by 6 persons under tutelage of renowned histopathologists (Prof. Obioma Okechi of Abia State University and Prof. O.G. Avwioro of Delta State University) according to the method and check list of Walker (2006). The check lists for reporting the intensity and clarity of the stained slides were as follows:
(i) Deeply stained and clear, optimal nuclear staining observed
(ii) Moderately stained, clear, moderate nuclear staining observed
(iii) Lightly stained, slightly clear, slight nuclear staining observed
(iv) Very lightly stained, lacks clarity, no nuclear staining

The category with the highest observed frequency was reported for every stained slide

Microscopy and photomicrography

The sections were viewed and photomicrograph, using Leica Binocular Microscope DM 1000 in-built digital camera.

Photomicrograph of pathological and normal tissues stained with dyes extracted from the various plant materials were taken, using x 100 magnifications and printed by an inkjet printer. Eroschenko (2013) provided a guide for the labeling of the photomicrographs.

RESULTS

Results from phase one (staining with freshly prepared plant extracts alongside with haematoxylin) indicated that 100mg/ml was the most effective concentration of the plant products in the staining of tissues. Lower concentrations (75 to 25 mg/ml) produced very faint colouration as shown in Plate 1 for C. longa. *C. ferruginea* extracts (both ethanolic and aqueous solutions) as counter stain produced faintly stained pale red coloration of the cytoplasm (Plates 2 and 3). Tissue sections stained with *Garcinia kola* pod extracts as a counter stain showed fairly good cyto-architectural distinctions, that is the nuclei and other organelles appeared fairly good and moderately stained (Plates 4,) when compared with the control sections stained with the conventional Haematoxylin and eosin method (Plates 5).

When each of the tissue slides was stained with only a single stain (mono staining)

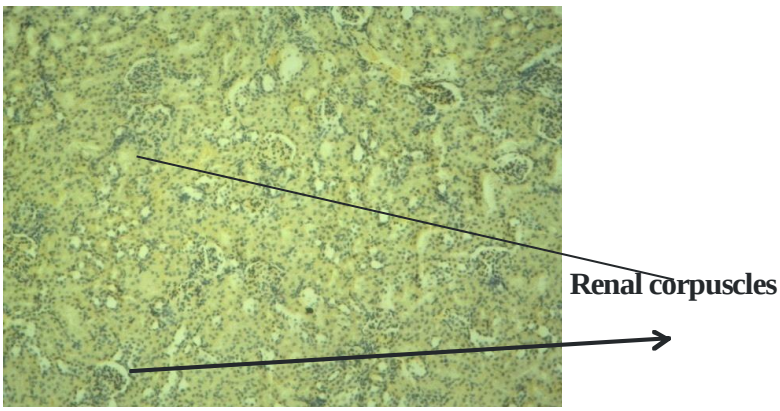

Plate 1. Normal kidney tissue stained with fresh aqueous *Curauma longa* (turmeric) staining solution as a counter stain. (Yellowish green cytoplasm).

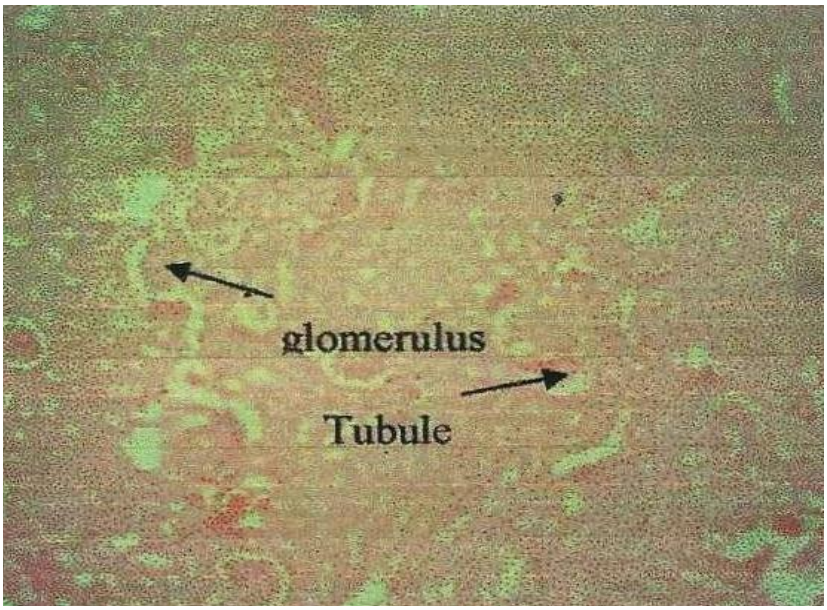

Plate 2. Normal kidney tissue stained with fresh aqueous Cnestis extract to show faintly stained pale red cytoplasm and good nuclear staining (x100).

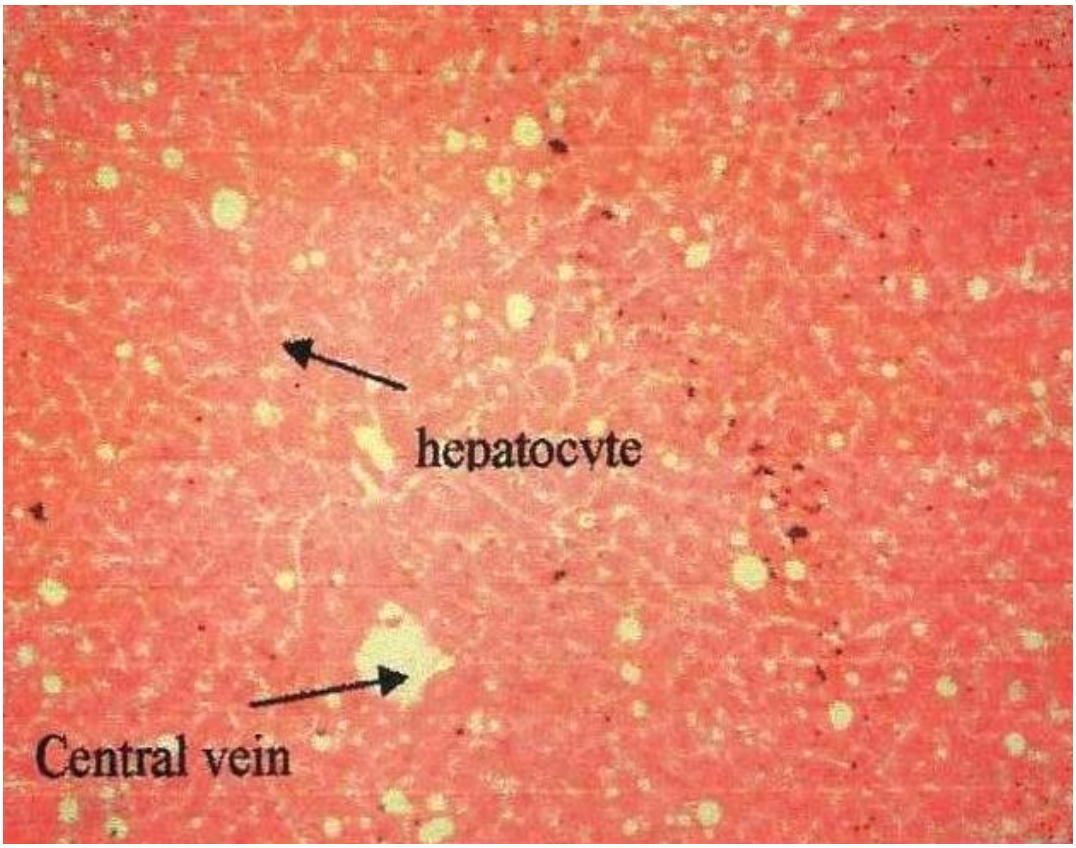

Plate 3. Normal liver tissue stained with fresh ethanolic Cnestis extract to show fainty stained pale red cytoplasm and good nuclear staining (x100).

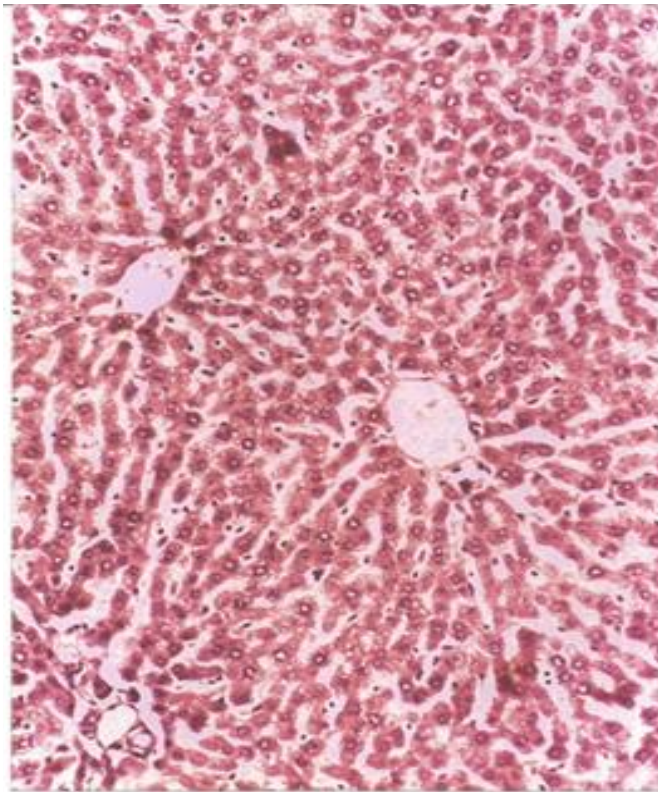

Plate 4. Normal liver tissue stained with fresh *Garcinia kola* pod extract to show fairly good cyto architectural distinctions (x200).

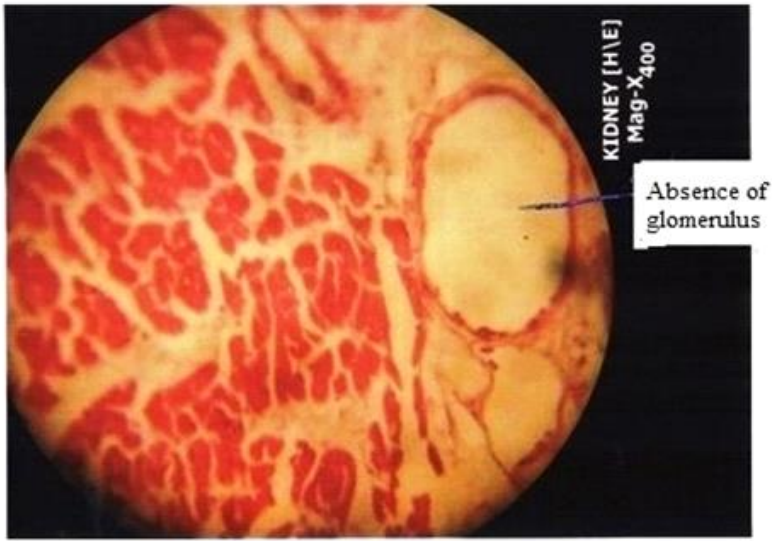

Plate 5. Pattological kidney tissue slide stained with haematoxylin and eosin. Agromerular kidney discerned.

irrespective of the form and type of the plant extract, there was a drastic reduction in the intensities and clarity of features. With the exception of *C.longa* which lightly stained the pathological liver and kidney tissues with slight clarity of features as a mono stain, the remaining plant products lightly stained the tissues without clarity of features as mono stains (Table 1).

After staining tissue slides with 6-month old plant extracts, reduction in the intensities and clarity of features were observed when compared with freshly prepared extracts (Table 2). In summary, freshly prepared plant extracts alongside with either haematoxylin or eosin gave the best staining result in the study. The second most potential staining reaction was given by the single (mono) staining, with each of the plant products alone. The 6-month old plant extracts alongside with haematoxylin gave the poorest staining result.

Table 1. Staining intensity and clarity of freshly prepared plant extracts as mono stains.

Solution	Result
Ethanolic *Curcuma* extract with mordant	Lightly stained, slightly clear, slight nuclear staining observed
Ethanolic *Curcuma* extract without mordant	Lightly stained, slightly clear, slight nuclear staining observed
Aqueous *Curcuma* extract with mordant	Lightly stained, slightly clear, slight nuclear staining observed
Aqueous *Curcuma* extract without mordant	Lightly stained, slightly clear, slight nuclear staining observed
Ethanolic *Cnestis* extract with mordant	Very lightly stained, lacks clarity, no nuclear staining
Ethanolic *Cnestis* extract without mordant	Very lightly stained, lacks clarity, no nuclear staining
Aqueous *Cnestis* extract with mordant	Very lightly stained, lacks clarity, no nuclear staining
Aqueous *Cnestis* extract without mordant	Very lightly stained, lacks clarity, no nuclear staining observed
Ethanolic *Garcinia* extract with mordant	Very lightly stained, lacks clarity, no nuclear staining
Ethanolic *Garcinia* extract without mordant	Very lightly stained, lacks clarity, no nuclear staining
Aqueous *Garcinia* extract with mordant	Very lightly stained, lacks clarity, no nuclear staining
Aqueous *Garcinia* extract without mordant	Very lightly stained, lacks clarity, no nuclear staining

The observations were conducted by 6 persons according to the method of Walker (2006) and supervised by Prof. O.O. Okechi of the Department of Medical Laboratory Science, Abia State University and Prof. O.G. Avwioro of Delta State University, Nigeria. *C. longa, C. ferruginea* and *G. kola* were used as secondary (counter) stains stain, instead of eosin.

Table 2. Staining intensity and clarity of 6 month old plant extracts alongside eosin.

Solution	Result
Ethanolic *Curcuma* extract with mordant	Moderately stained, clear, no nuclear staining
Ethanolic *Curcuma* extract without mordant	Moderately stained, clear, moderate nuclear staining observed
Aqueous *Curcuma* extract with mordant	Moderately stained, clear, moderate nuclear staining observed
Aqueous *Curcuma* extract without mordant	Moderately stained, clear, moderate nuclear staining observed
Ethanolic *Cnestis* extract with mordant	Very lightly stained, lacks clarity, no nuclear staining
Ethanolic *Cnestis* extract without mordant	Very lightly stained, lacks clarity, no nuclear staining
Aqueous *Cnestis* extract with mordant	Very lightly stained, lacks clarity, no nuclear staining
Aqueous *Cnestis* extract without mordant	Very lightly stained, lacks clarity, no nuclear staining
Ethanolic *Garcinia* extract with mordant	Lightly stained, slightly clear, slight nuclear staining observed
Ethanolic *Garcinia* extract without mordant	Lightly stained, slightly clear, slight nuclear staining observed
Aqueous *Garcinia* extract with mordant	Lightly stained, slightly clear, slight nuclear staining observed
Aqueous *Garcinia* extract without mordant	Lightly stained, slightly clear, slight nuclear staining observed

DISCUSSION

Cellular structures are selectively stained with various natural and synthetic dyes. Some of these tissue structures require combination of stains to demonstrate their presence. Acidity, alkalinity and mordant have been reported to affect some stains. *C. longa* (turmeric) was used as a counter-stain in the study instead of the conventional eosin. A counter-stain is a stain with color contrasting the primary stain making the stained structure more readily visible (Bassey et al., 2011). Tumeric imparted a yellowish green coloration to the cytoplasm, which contrasted satisfactorily with the blue/violet color of the nucleus. This finding is in consonance with that of Avwioro et al. (2007) which who stated that crude extracts of *C. longa* (Tumeric) stained collagen fibres, cytoplasm and red blood cells intense yellow in fashion similar to eosin, suggesting that turmeric has the best staining potential than any of the plant products studied in this work. This was probably due to the acidic nature of the dye. Previously, Bassey et al. (2011) had obtained similar results on staining of tissue with tumeric. Phytochemical screening of the turmeric dye confirmed the presence of saponins, tannins, flavonoids and alkaloids. Saponins are known to reduce surface tension and this also enhances staining (Chukwu et al., 2011). Flavonoids on the other hand are primarily responsible for the yellow pigments.

Haematoxylin in combination with eosin (a synthetic dye) is used for the demonstration of general tissue structures such as muscle fibre, connective tissues etc. in histology. Nuclei of cells take up the haematoxylin stain and they appear dark violet/blue in color. Cytoplasm or red blood cells take up eosin and appear pink. In the present study, the tissues, which were stained with H&E, showed good nuclear and cytoplasm differentiation as the nucleus stains dark violet/blue and the cytoplasm pink/red. There was no obscurity to the nucleus caused by addition of the counter-stain and the tissue morphology was clearly shown. Tissue sections stained with *C. ferruginea* extract as a counter stain showed good nuclear staining but very pale cytoplasm staining, giving poor nuclear cytoplasm differentiation. The observed good nuclear staining was probably due to the earlier application of haematoxylin as a primary stain to the same slide. An attempt to increase the concentration of *C. ferruginea* extract beyond 100mg/ml in order to improve cytoplasm staining in the study rendered nuclear staining obscure. This suggests that high concentration of *Cnestis* dye solution renders it less valuable as a possible source of good histological stain.

The study on the *G. kola* pod extract as a possible alternative stain to eosin on the tissue slide suggests that the stain may have the capacity to stain the cytoplasm (basic) component of the tissue cells. The extract appeared red in colour and seemed to act as an acidic stain, which binds to acidophilic substances. Nevertheless, it should not be allowed to stay longer as eosin; the period of staining should be reduced, so as to avoid discoloration of the nuclei. Also, its pH must be monitored in view of the high acidic content of

the extract in order to avoid discoloration of the nuclei. With the exception of *C. longa* which lightly stained the pathological liver and kidney tissues with slight clarity of features as a mono stain, the remaining plant products lightly stained the tissues without clarity of features as mono stains. This suggests that the plant products used alone may not act as good histological stains. The 6 month old extracts gave significantly reduced staining intensities and clarity of normal and pathological liver and kidney features when compared with freshly prepared extracts. This observation may be due to deterioration of the plant products after 6 months of extraction.

CONCLUSSION AND RECOMMENDATIONS

Although eosin is the most utilized counter-stain in histology, turmeric dye obtained from *C. longa* extract may serve as a complementary stain to it. Further studies should be done to show how the cytoplasm and nuclear staining characteristics of the plant products could be enhanced.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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