International Journal of Biochemistry Research & Review

29(6): 26-33, 2020; Article no.IJBCRR.55114
ISSN: 2231-086X, NLM ID: 101654445

Study of Staining Capability of Alcoholic and Aqueous *Hibiscus sabdariffa* Extract in Demonstration of Selected Fungal Moulds in Some Selected Tissue Section of Wistar Rat

S. Y. Ma'aruf[1], M. O. Mohammed[1], O. G. Avwioro[2], A. T. Muhammad[1], R. I. Tsamiya[1], U. Abubakar[1*], I. Mohammed[1], A. Umar[1], S. M. Sani[1], H. Kabir[1], B. A. Bello[1], S. D. Abubakar[3], F. A. Dogondaji[1], N. Okorie[4] and S. Garba[5]

[1]Department of Histopathology, School of Medical Laboratory Science, Usmanu Danfodiyo University, Sokoto, Nigeria.
[2]Department of Anatomy, Delta State University, Delta State, Nigeria.
[3]Department of Medical Laboratory Science, Ahmadu Bello University, Zaria, Nigeria.
[4]Department of Medical Laboratory Science, Faculty of Health Sciences, Ebonyi State University, Nigeria.
[5]Department of Medical Laboratory Science, College of Health Science and Technology, Jahun, Jigawa State, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. Author SYM own the research and initiated it. Author MOM design the research and arrange the work. Author ATM scrutinized the work and give beautiful suggestions. Author OGA overall manger of the research and eliminate all mistakes. Author RIT did statistical analysis of the data gathered. Author UA carried out photomicrographs and take care of the animals. Author IM carried out dissection of the rats and harvested the organs used for this research. Author AU did literature review and authors BAB and SMS source the materials for the work. Author HK arranged references. Author SDA cross check the statistics. Authors FAD and NO carried out staining procedures and screened the slides and author SG arranged the slides for photomicrographs. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IJBCRR/2020/v29i630193
Editor(s):
(1) Halit Demir, Yil University, Turkey.
Reviewers:
(1) Dennis, Amaechi, Veritas University, Nigeria.
(2) Kanti Shrestha, Nepal Academy of Science and Technology, Nepal.
(3) Gabriela Dumitru, Alexandru Ioan Cuza University, Romania.
Complete Peer review History: http://www.sdiarticle4.com/review-history/55114

Original Research Article

Received 04 January 2020
Accepted 10 March 2020
Published 24 June 2020

**Corresponding author: E-mail: uabubakar527@yahoo.com;*

ABSTRACT

Introduction: *Hibiscus sabdariifa* is a natural dye, generally called roselle. Most of Synthetic dye have been established to cause toxicity. The aim of this research was to determine the staining effect of different concentration of *Hibiscus sabdariffa* extracts at various pH, duration on fungal staining.
Methods: Standard Gomori's metenaine silver stains as control and *Hibiscus sabdariffa* extracts (alcoholic and aqueous) were used to stain a fungal (*Aspergillus spp*) positive lung tissue, using various concentration (5% and 10%) of the extract, with change of pH achieved by treating the extracts with ammonium hydroxide and glacial acetic acid at various duration (30 seconds and 1 hour respectively), each was used for staining *Aspergillus spp* in tissue section as a primary stain replacing (4% Chromic acid, sodium metabisulphite, Hexamine working silver solution and Sodium thiosulphate).
Results: All extracts after treatment were acidic, the fungi were best demonstrated with Aqueous hibiscus stains (5% untreated, 10% untreated and 5% glacial acetic acid treated) at longer duration, staining the capsule, light to dark-brown in a light-green background when compared with the alcoholic hibiscus stain. Few of the alcoholic hibiscus stains show metachromatic property. The Hibiscus stains stained better at longer duration and the change of pH was indirectly proportional to the staining ability of the hibiscus stains.
Conclusion: The results obtained indicate that hibiscus plant extract have the potential for use in the morphological identification of fungi in formalin-fixed paraffin-embedded tissue section.

Keywords: Hibiscus sabdariffa extract; alcohol and aqueous extract; histological section; fungal staining.

1. INTRODUCTION

Dyes are chemical substances of chemical or synthetic origin, soluble in a medium used to impart a desired color to a non food material like paper, leather, wood, textile and even cosmetics in a process known as dying [1]. The importance of color in textile products cannot be under estimated. Colors have fascinated mankind since the down of civilization. Since the introduction of synthetic dyes in the year 1856 the use of natural dye was on the wane [2]. Some dyes require the addition of mordants, oxidants, accelerators and adjustment of pH before they can stain tissues while others do not require these substances in other for them to stain tissues. That is, simple aqueous or alcoholic solutions of the dyes can be used as stains, generally called simple stain [3,4].

The standard reference work used for the identification of dyes is the color index, which first appear in 1923. The Color Index is published jointly by the Society of Dyers and Colorists and the American Association of Textile Chemists and Colorists. A constitution number (CI No. or Color Index number) is allocated when the chemical constitution of a dye is known and dyes of similar constitution but different trade names receive the same Color Index number [5]. The dye can be synthetic or naturally sourced, Synthetic stains are derivatives of the hydrocarbon (C_6H_6) which is represented by benzene ring [6], while Natural stains are dyes that have been from plant or animal source, historically plants have been used for the extraction of a majority of dyes [7]. Microbial stains are used to impart color in order to make the cells and tissues more distinct. Although, microorganisms can be seen with the aid of a light microscope, they need to be fixed and or stained to increase visibility, accentuate morphological features and sometimes preserve them for further study [1] e.g Gram'stain, Gridley Allen stain, Lactophenol blue stain.

Over the past many years, it has been observed that synthetic dyes have many disadvantages associated with them like toxicity, pollution, allergenicity etc. but natural dyes have no such disadvantages. So because of growing disadvantages of synthetic colors, people started using natural colors [8]. Also many developing countries can no longer afford the ever increasing cost of synthetic dyes; the use of cheaper, naturally occurring dyes from plants is being viewed as an alternative to synthetic dyes [9].

Roselle is botanically called *Hibiscus sabdariffa* Linn (family Malvaceae) [10]. There are two types of true roselle (*Hibiscus sabdariffa*) which are *H. sabdariffa var. altissima* and *H. sabdariffa var. Sabdariffa* [11].

The calyx in focus, *Hibiscus sabdariffa* is commonly available with no hazardous threat to the humans and it environment. This research intends to study the staining property of alcoholic and aqueous *Hibiscus sabdariffa* extract in the demonstration of selected fungal mould in tissue sections.

2. MATERIALS AND METHODS

2.1 Study Design/Area

This research is an experimental design. The study was carried out in the Department of Histopathology. School of Medical Laboratory Science, Usmanu Danfodiyo University, Sokoto. North – Western Nigeria.

2.2 Choice of Fungi for Staining

A fungal positive (*Aspergillus spp*verified) tissue block (paraffin wax processed) was obtained sequel to necessary protocol from the Department of Histopathology, Usmanu Danfodiyo University Teaching Hospital (UDUTH), Sokoto.

2.3 Plant

Roselle calyx (*Hibiscus sabdariffa*) were purchased in a local market in Sokoto and identified in the Department of Pharmacognosy and Ethnopharmacy, Faculty of Pharmaceutical Sciences of Usmanu Danfodiyo University, Sokoto, Nigeria and a voucher specimen was deposited at the departmental herbarium (PCG/UDUS/Malv/0001) for identification (*H. sabdariffa* L). The calyx of roselle brought was rinsed and dried, and then was ground to powdery form using a blender (Sonik® Japan SB-735), sieved and stored in a dry container.

2.4 Preparation of Hibiscus Stains

Alcohol extract: Alcoholic extract was obtained by refluxing 5 g and 10 g of the dry mill calyx weighed with a sensitive balance, each dissolved in 100 ml of 80% ethanol for 4 hours, and then was filtered [12] with filter paper, then transferred into six reagent bottles and Four were respectively treated with 0.5ml of Glacial acetic acid and Ammonium hydroxide, while two remain untreated. Thymol was added and the whole contents were mixed, corked and labeled appropriately [1].

Aqueous extract: Aqueous extract was obtained by dissolving 5 g and 10 g of the dry mill calyx into 100 ml boiling water, then was mixed, agitated and allowed to stand for 40 min, filtered [13] with filter paper and transferred into six reagent bottles and treated as that of the alcohol extract.

2.5 Staining Procedures

Control fungal staining: The sections for fungal stains were deparaffinized and hydrated to distilled water through 100%, 90%, 70% alcohol for 2 minutes each, it was then stained according to Gomori's methenamine silver staining by first oxidizing in 4% Chromic acid for 30 minutes and rinsed in water. The sections were then bleached in 1% Sodium metabisulfite for 1 minute and rinsed with water. The sections were treated in pre-heated Hexamine working silver solution at 56^{o}c in incubator until the sections appear paper bag brown and then were rinsed with water.

The sections were further treated with Sodium thiosulphate for 5 minutes, rinsed with water and counter stained in light green solution for 1 min, section were rinsed with water, dehydrated, in ascending grade of alcohols, cleared in xylene and coverslipped with DPX.

Experimental fungal staining: The sections of fungal stain were deparaffinized and hydrated. Section were stained following Gomori's methenamine silver staining (using untreated and treated 5% and 10% *Hibiscus sabdariffa* alcoholic and aqueous extracts differently on sections as primary stain, replacing chromic acid, sodium metabisulfite, hexamine working silver solution and sodium thiosulphate) for 30 seconds and 60 minutes respectively. The sections were counter-stained with Light green solution for 1 minute each, dehydrated, cleared and cover slipped.

Photomicrograph grade and score: Each of the photomicrographs for each control and extracts-stained tissue were graded and scored according to their intensity on the target (fungi and bacteria) and background (tissue elements) in line with an establishment in a research.

3. RESULTS

3.1 Extraction

The boiled water and 80% ethanol were good in the extraction of the *Hibiscus dye.* The hibiscus

powder dissolves slowly in boiled water and dissolves quickly in 80% ethanol. The color of the untreated aqueous and untreated alcoholic extracts is red and mauve respectively. The intensity of untreated extracts concentration is indicated in (Table 1). Treatment with glacial acetic acid did not change the color of any solution, however, the color of all the hibiscus extract changed to dark color on each added drop (0.05 µl) of ammonium hydroxide, but it remained uniform after thorough mixture. The pH of each treated extract was also determined using litmus paper, which all indicates acidity (Table 1).

3.2 Staining Details

The various results show that aqueous hibiscus stains used shows better staining potential on fungal staining than alcoholic extracts bacterial staining, also hibiscus stains used at 30 seconds show less staining potential when compare with that for 60 minutes. Untreated 5% aqueous, 5% glacial acetic acid treated aqueous hibiscus stains and 10% untreated aqueous hibiscus stains, both at 60 minutes shows better staining potential of the extracts on fungi staining the fungi spores and hyphae (Figs. 2a, 2b and 3a), hence scored 3 when compared with that of Control (Fig. 1) that was scored 4. It was also noticed that 5% untreated alcohol hibiscus stains, 5% ammonium hydroxide treated alcohol hibiscus stains and 10% ammonium hydroxide treated alcoholic hibiscus stains both at 60 minutes give meta-chromatic stain (Figs. 4a, 5a, 5b) in fungal staining the fungi brown, alveolar cells purplish and red blood cells golden-brown (Table 2).

Base on the concentration of the Hibiscus stains, the 5% hibiscus stains show greater staining capabilities than the 10% hibicus stains. The pH variations of the aqueous hibiscus stains have a strong impact on the staining potential; the lower the pH, the higher the staining potential. The aqueous hibiscus stains at longer duration give better staining results when compared with the shorter duration.

There was no significant changes in the staining capabilities of glacial acetic acid and ammonium hydroxide treated alcoholic hibiscus stains which means pH have partial impact on the alcoholic hibiscus stains but it was observe that the longer duration also have a strong impact on the staining capabilities of the alcoholic hibiscus stains as indicated in Table 2.

4. DISCUSSION

The hazardous effects of synthetic dyes to the environment have led to a reverse approach by researchers to search and use local natural dyes to reduce dependence and limit the use of synthetic dyes.

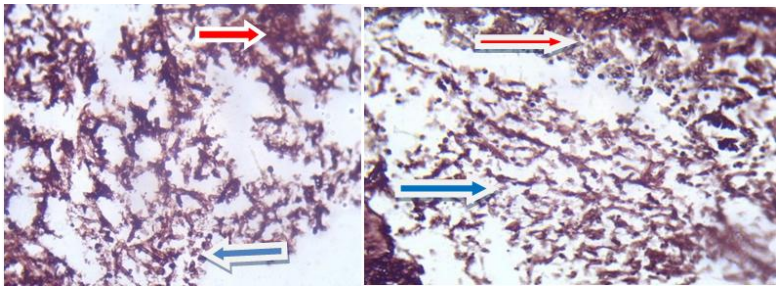

Fig. 1. Conventional GMS stained lung tissue as control (X400)

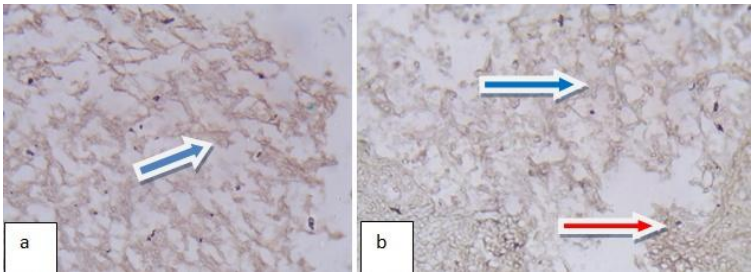

Fig. 2. (a) 5% aqueous H.S. stain used to stain fungi positive lung tissue for 60 minutes (b) 5% aqueous H.S. stain-glacial acetic acid extract used to stain fungi positive lung tissue for 60 minutes (X400)

LEGEND: Blue arrow = showing Fungi mould, Red arrow = showing interstitial tissue

Table 1. Physical character of the extracts and pH reaction of extracts

S/N	Extract	Colour	Intensity	pH reaction
A 5% Hibiscus in Boiled Water				
1	untreated	RED	+	RED
2	Glacial Acetic Acid Treated	RED	+	RED
3	Ammonium Hydroxide Treated	RED	+	RED
B 10% Hibiscus in Boiled Water				
4	Untreated	RED	+	RED
5	Glacial Acetic Acid Treated	RED	++	RED
6	Ammonium Hydroxide Treated	PURPLE-RED	++	RED
C 5% Hibiscus in 80% Ethanol				
7	Untreated	MAUVE	+	RED
8	Glacial Acetic Acid Treated	MAUVE	+	RED
9	Ammonium Hydroxide Treated	MAUVE	++	RED
D 10% Hibiscus in Boiled Water				
10	Untreated	MAUVE	+++	RED
11	Glacial Acetic Acid Treated	MAUVE	+++	RED
12	Ammonium Hydroxide Treated	MAUVE	+++	RED

+; Light color, ++; Moderate color, +++; Deep color

Table 2. Grading and scoring of the extracts staining intensity on fungi and bacteria

S/N	Extracts	Treatment	Time	Lungs (Fungi)	
				Grade	Score
1	Control			++++	4
2	5% aqueous	Untreated	30 seconds	+	1
3		Untreated	60 minutes	+++	3
4		Ammonium Hydroxide	30 seconds	+	1
5		Ammonium Hydroxide	60 minutes	++	2
6		Glacial acetic acid	30 seconds	+	1
7		Glacial acetic acid	60 minutes	+++	3
8	10% aqueous	Untreated	30 seconds	+	1
9		Untreated	60 minutes	+++	3
10		Ammonium Hydroxide	30 seconds	-	0
11		Ammonium Hydroxide	60 minutes	++	2
12		Glacial acetic acid	30 seconds	+	1
13		Glacial acetic acid	60 minutes	++	2
14	5% alcohol	Untreated	30 seconds	+	1
15		Untreated	60 minutes	++	2[a]
16		Ammonium Hydroxide	30 seconds	+	1
17		Ammonium Hydroxide	60 minutes	++	2[a]
18		Glacial acetic acid	30 seconds	+	1
19		Glacial acetic acid	60 minutes	+	1
20		Untreated	30 seconds	+	1
21		Untreated	60 minutes	+	1
22		Ammonium Hydroxide	30 seconds	+	1
23	10% aqueous	Ammonium Hydroxide	60 minutes	++	2[a]
24		Glacial acetic acid	30 seconds	+	1
25		Glacial acetic acid	60 minutes	++	2

-; Undefined and unstained, +; Undefined and stained, ++; Define and stained, +++; Well defined and stained, ++++; Very well defined and well stained, [a]; Metachromatic

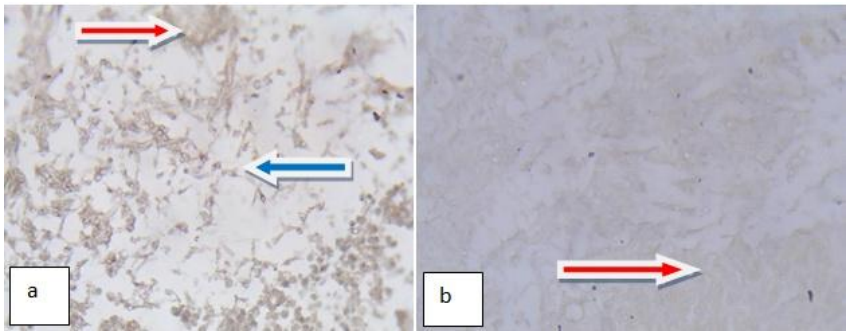

Fig. 3. (a) 10% aqueous H.S. stain used to stain fungi positive lung tissue for 60 minutes. (b) 10% aqueous H.S. stain-ammonium hydroxide treated used to stain fungi positive lung tissue for 30 seconds (X400)

Legend: Blue arrow = showing Fungi mould, Red arrow = showing interstitial tissue

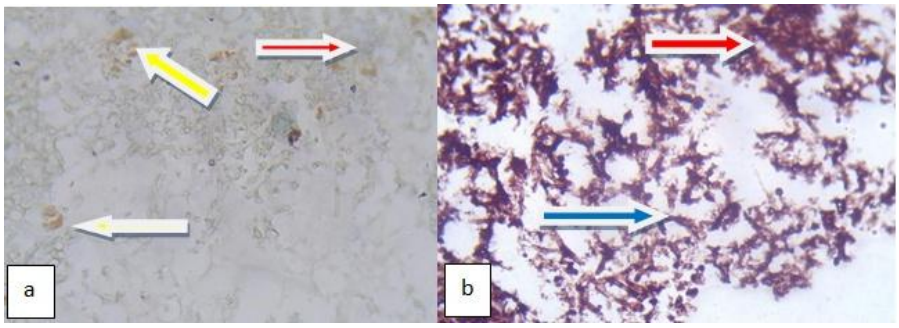

Fig. 4. (a) 5% alcohol H.S. stain-ammonium hydroxide treated used to stain fungi positive lung tissue for 30 seconds. (b) Conventional GMS stained lung tissue as control. (X400)

Legend: Blue arrow = showing Fungi mould, Red arrow = showing interstitial tissue, Yellow arrow = showing stains red blood cells

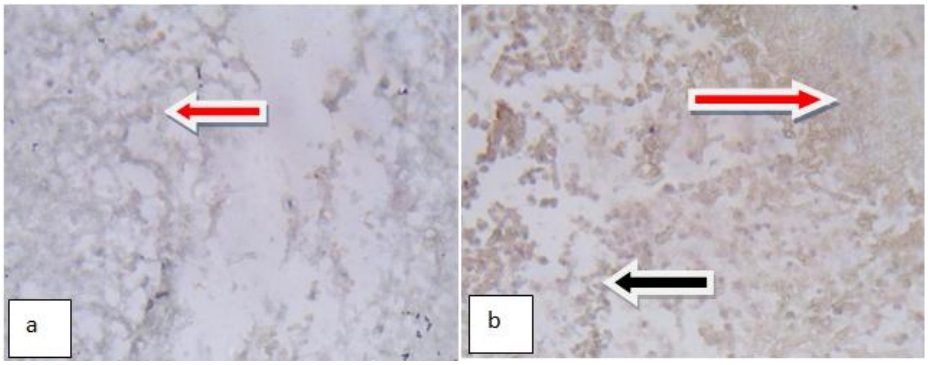

Fig. 5. (a) 5% alcohol H.S. glacial acetic acid treated extract used to stain fungi positive lung tissue for 60 minutes. (b) 10% alcohol H.S. glacial acetic acid treated extract used to stain fungi positive lung tissue for 60 minutes. (X400)

Legend: Blue arrow = showing Fungi mould, Black arrow = showing variable stained cellular element

In line with many recommendations, to our knowledge this study is the first to be conducted on tissue section whereby *Hibiscus sabdariffa* at different concentration, pH, solvent and staining duration was used as fungal stain, though it have been attempted by some researchers [1,14] to stain smears.

In this study, it was observed that the solubility of *Hibiscus sabdariffa* powder as established in this

research work comply with the result established by Omorodion and Achukwu [15].

Fungi growth was observed on boiled water residue on the filter paper during filtration, this is in line with a report made in a research work [1]. But this was resolved by adding thymol to the extracts. After changing the pH of the extracts in this work using a high quantity of ammonium hydroxide and glacial acetic acid than the quantity used in a research [1] to demonstrate *Aspergillus spp* using different hibiscus extracts stains, the result obtained in this research still indicate that the extracts are in acidic state which might be due to high acidity of *Hibiscus sabdariffa* which is concomitant with some research works [16, 9,17,18]. While in a Deepali et al. [13] research, Hydrochloric acid was used to acidify the extracts in staining fungi and paramecium different from the approach used in the research and another research work [1].

In this study, different concentrations (5% and 10%) were used. The result obtained for fungal staining show that 5% untreated, 10% untreated and 5% glacial acetic treated aqueous extract, show better staining effect on fungi which technically signifies that 5% concentration have significant staining ability than 10% concentration with the fungi capsules stained light to dark-brown in a light green background, which support some research works [19,20], though there works were on tissue structure and not microorganism, which may be due to the method of extraction and modification used in this method.

Also in this study, extract that are untreated and treated with glacial acetic acid give a good fungal staining ability as scored and shown in the photomicrographs, staining the fungi capsule light to dark-brown within the light-green tissue background than the extracts treated with ammonium hydroxide, this support the some research studies [1,18], which means change of pH is indirectly proportional to the staining ability of the *Hibiscus sabdariffa* extracts.

It was observed in this work that staining duration at 60 minutes (longer duration) give better staining duration than the 30 seconds (shorter duration), this may be due to duration (1 minute) of the light green solution used as counter-stain that might have masked the staining effect of the *Hibiscus sabdariffa* extracts used at shorter duration which is concomitant with a research [20].

Base on the type of solvent used in this work, the aqueous extracts give a better fungal spore staining ability (*Aspergillus* spp) than the alcoholic extracts, these results are concomitant with a work [1,14] but oppose Ihuma et al. [18] work and this may be due to mordanting and high percentage of glacial acetic acid used in their work. It was observed that tissue stained for fungal staining with five percent alcoholic extracts treated with ammonia were observed to give a varying coloration on the tissue cellular elements; this might be due to the theory of metachromatic staining [4].

It was observed that most of Hibiscus extracts have affinity with red blood cell (rbcs); the alcoholic extracts stain the erythrocytes golden-brown while the aqueous stain the erythrocytes dark-brown.

5. CONCLUSION

The aqueous Hibiscus stain shows more staining ability on fungi than alcoholic Hibiscus stains at longer duration. The fungi were well and best demonstrated with 5%, 10% untreated and 5% glacial acetic acid treated aqueous extract both at longer duration among all extracts staining the capsule, light to dark-brown in a light-green background when compared with demonstration of bacteria. Few of the alcohol extracts show metachromatic property. The best results obtained signify that *Hibiscus sabdariffa* extracts could be used in diagnostic mycology.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Braide W, Akobundu C, Nwaoguikpe RN and Njiribaeko LC. The use of extracts from four local Nigerian plants for the staining of selected bacteria and moulds. Afr. J. Microbiol. Res. 2011;5:78-86.
2. Kodrikar P. Probems and aspects of natural dyes [internet]; 2018.
(Accessed March, 2018)
Available:http://www.fibre2fashion.com/industry-article/4748/problems-and-aspects-of-natural-dyes

3. Avwioro OG, Aloamaka PC, Ojianya NU, Oduola T, Ekpo OE. Extracts of pterocarpus osun as a histological stain for collagen fibres. Afr. J. Biotechnology. 2005;4:460-462.
4. Avwioro OG. Histochemistry and tissue pathology. Delta: Claverianum Press. 2010;134-260.
5. Baker FJ, Silverton RE and Pallister CJ. Baker and Silverton's introduction to medical Laboratory Technology. Nigeria: Bounty Press Limited. 2001;220-242:385-390.
6. Ochei J, Kolhatkar A. Medical laboratory science theory and practice. India, New Delhi. Tata McGraw-Hill Publishing Company Ltd. 2000;471-479.
7. Grifoni D, Bacci L, Zipoli G, et al. The role of natural dyes in the UV protection of fabrics made of vegetable fibres. Dyes Pigm. 2011;91:279-85.
8. Rubia, Bharwaj A. A review: Natural colourants. Int. J. Eng. Sci. Res. Technol. 2016;5:778-781.
9. Chinyere CG, Etoforinini CC. Alternative staining of kidney tissue sections with extracts of tumeric (*Carcuma longa*) and Zobo (*Hisbiscus sabdariffa*). J. Histotechnol. 2017;1-9.
10. Morton JF. Fruits of warm climates. Florida Flair Books; 1987.
11. Smith NM. Weeds of the wet/dry tropics of Austalia - A field guide. Enviroment Center NT, Inc. 2002;112.
12. Sridhara SU, Raju S, Gopalkrishna AH, et al. Hibiscus: A different Hue in Histo-pathology. J. Med. Rad. Path. Sur. 2016; 2:9-11.
13. Ola MA, Abiola OO, Benard SA, et al. Hibiscus-Shorgum: A new morphological stain in neuro-histology. Int. J. Health Res. Inno. 2016;4(1):31-38.
14. Deepali K, Laleta S, Deepika M. Application of aqueous plant extract as biological stains. IJSER. 2014;5(2):1586-1589.
15. Omorodion NT, Achukwu P. Investigation of *Hibiscus sabdariffa* (Roselle) as Histological stain and in assessment of bar bodies. Am. J. Biomed. Sci. 2017;9(1):15-19.
16. Cheng CW, Saad SM, Abdullah R. Alternative staining using extracts from Hibiscus (*Hibiscus rosa-sinensis* L.) and red beet (*Beta vulgaris* L.) in diagnosing ova of intestinal nematodes (*Trichuris triciura* and *Ascaris lumbricoides*). European J. Biotechnol. Biosci. 2014;1(5): 14-18.
17. Hashim EA. The use of watery extract of kujarat flower *Hibiscus sabdariffa* as a natural Histological stain. Iraqi J. Med. Sci. 2006;5(1):29-33.
18. Ihuma JO, Asenge GH, Abioye JOK, Dick SK. Application of methanolic extracts from *Hibiscus sabdariffa* Linn as a biological staining agent for some fungal species. IJPAES. 2012;2(2): 254-259.
19. Ibnouf A, AbdulRaheem E, Seed Ahmed M, Dahab D. Assessment of staining quality of Roselle (*Hibiscus sabdariffa*) on formalin fixed paraffin-embedded renal tissue sections. Int. J. Curr. Res. Rev. 2014;6(21):26-28.
20. Raheem EMA, Ibnouf AO, Shingeray OH, Farh HJM. Using of *Hibiscus sabdariffa* extract as a natural histological stain of skin. Am. J. Res. Commun. 2015;3(5):211-216.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/55114