Microbiology Research Journal International

30(2): 26-33, 2020; Article no.MRJI.55046
ISSN: 2456-7043
(Past name: British Microbiology Research Journal, Past ISSN: 2231-0886, NLM ID: 101608140)

Staining Property of Alcoholic and Aqueous *Hibiscus sabdariffa* Extract in Demonstration of Selected Bacteria in Tissue Sections of Wistar Rats

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Authors' contributions

Author SYM own the research and initiated it. Author MOM designed the research and arranged the work. Author ATM scrutinized the work and gave the beautiful suggestions. Author OOO managed the overall research and eliminated all mistakes. Author RIT did the 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 the literature review. Authors BAB and SMS sourced the materials for the work. Authors HK and SG arranged the references. Author SDA cross checked the statistics. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/MRJI/2020/v30i230195
Editor(s):
(1) Prof. Nermeen Mohamed Bahi El-Deen El-Shaffie, National Research Center, Egypt.
Reviewers:
(1) Benard Solomon Adebayo, University of Ilorin Teaching Hospital, Nigeria.
(2) Sowjanya Pulipati, Vignan Pharmacy College, India.
Complete Peer review History: http://www.sdiarticle4.com/review-history/55046

Received 29 December 2019
Accepted 05 March 2020
Published 04 April 2020

Original Research Article

ABSTRACT

Introduction: Dye is used for the artificial colouration of a substance to facilitate its examination by the use of the coloured organic molecule, a process called staining. Over the past many years, it has been observed that synthetic dyes have many disadvantages associated with them like toxicity and allergenicity. This study aimed to determine the staining effect of *Hibiscus sabdariffa* extracts

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of various concentration, various pH and duration in bacteria staining compared with Gram's staining.
Methods: Standard Gram stains as control and both *Hibiscus sabdariffa* extracts (alcohol and boiled water) were used to stain inflammatory appendix tissue and lung tissue inoculated with bacteria using various concentration (5% and 10%), at various duration (30 seconds and 1 hour) and with change of pH, achieved by treating the extracts with ammonium hydroxide and glacial acetic acid. Each was used as a counterstain in bacterial stain (to replace Safranin/Neutral red) Safranin/Neutral red.
Results: All extracts after treatment were acidic but the change of pH was indirectly proportional to the staining ability of the extracts. The Hibiscus solutions gave the background, a brown colouration. And inflammatory cells were demonstrated better than the bacteria with aqueous Hibiscus solution strongly when compared with the alcoholic Hibiscus solution.
Conclusion: The results obtained indicate that Hibiscus solution has the potential for use in diagnostic bacteriology in formalin-fixed paraffin-embedded tissue section.

Keywords: Hibiscus sabdariffa plant; alcohol and aqueous extract; tissue section and bacteria staining.

1. INTRODUCTION

Dyes are chemical substances of chemical or synthetic origin, soluble in a medium used to impart a desired colour to a non-food material like paper, leather, wood, textile and even cosmetics in a process known as dying [1]. Therefore there are two types of dyes, natural dyes and synthetic dyes [2]. Natural dyes are mainly extracted from natural resources. These are from renewable sources, while synthetic dyes are prepared from various chemicals, manmade materials etc. Dyes are also referred to as stains and can be used to add colour to tissues and microbes to make them optically distinct [1].

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. Microbial strains are used to impart colour 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].

Example of the Microbial strains are Gram's stain [3], Acid Fast Bacilli stain for bacteria, Gridley Allen stain, Grocott's modification of Gomori's methamine silver stain [4], lactophenol cotton blue stain for Fungi [1] Mann's methyl blue- eosin for Negri bodies [5], Hage-Fontana Silver method [4] and Levaditi's method for *Spirochaetes* [5].

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 colours, people started using natural colours [6].

Roselle is botanically called *Hibiscus sabdariffa* Linn (family Malvaceae) [7]. The calyx in focus, *Hibiscus sabdariffa* is commonly available and with no hazardous threat to the humans and its environment. This study (examines) the staining property of alcoholic and aqueous *Hibiscus sabdariffa* extract in the demonstration of some microorganisms 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 Experimental Animal

Two (2) Wistar rats were used for this research, which was purchased and kept in a well-ventilated metal cage in an animal house. Animals were anaesthetized using chloroform vapour. Longitudinal abdominal incision, subsequently with medial thoracic incision was made to carefully harvest the lungs. These organs harvested were then washed with normal saline and was transferred into bacteria-positive inoculated thioglycollate broth.

2.3 Choice of Microorganism for Staining

The Wistar rat lungs were incubated overnight in bacteria-positive inoculated thioglycollate broth of *Staphylococcus* spp [8,9] which was confirmed by biochemical test in Department of Medical Microbiology, Usmanu Danfodiyo University Teaching Hospital (UDUTH), Sokoto. It was fixed and processed histologically. Also, an appendix-bacteria positive tissue block (paraffin wax processed) was obtained from the Department of Histopathology, UDUTH.

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 (bought) 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 Plant Extracts

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 [10] with filter paper, then transferred into six reagent bottles and Four were respectively treated with 0.5 ml of Glacial acetic acid and Ammonium hydroxide, while two remain untreated. Thymol was added for preservation and the whole contents were mixed, corked and labelled appropriately [1].

Aqueous extract: The 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 [11] with filter paper and transferred into six reagent bottles and treated as that of the alcohol extract.

2.5 Staining Procedures

Control bacterial staining: The sections for bacterial stain were deparaffinized and hydrated. Then the sections were stained according to Gram staining technique with Crystal violet for 1 minute and then treated with Gram's iodine for 1 minute and rinsed with water. The section was decolourized with Acetone-alcohol with immediate attempt and washed with water and counterstained in Safranin solution for 1 minute and rinsed in water, dehydrated, cleared and coverslipped.

Experimental bacterial staining: The sections for bacterial stain were deparaffinized and hydrated. The Gram staining procedure was followed but replacing Safranin solution with untreated and treated 5% and 10% *Hibiscus sabdariffa* alcoholic and aqueous extracts differently on sections for 30 seconds and 60 minutes respectively, dehydrated, cleared and coverslipped.

Photomicrograph gradind and scoring: Each of the photomicrographs for each extract was graded and scored according to their intensity on the target (fungi and bacteria) and background (tissue elements) in line with Braide et al. [1] degree of intensity.

3. RESULTS

3.1 Extraction

The colour intensity of each untreated extract concentration is indicated in (Table 1). Treatment with glacial acetic acid did not change the colour of any concentration, however, the colour of all the hibiscus extract changed to a dark colour on each added drop (0.05 µl) of ammonium hydroxide, but it remained uniform after thorough mixture (Table 1). The 10% extracts show higher intensity than the 5% extracts. The pH of each treated extract was also determined using litmus paper, which all indicates acidity.

3.2 Staining Details

The various results show that the extracts show staining potential on bacteria staining technique although the extract demonstrated inflammatory cells (Fig. 3a) better than bacteria. The extract used at 30 seconds shows less staining potential when compared with that for 60 minutes. Aqueous extracts show considerably staining potential on the tissue than the alcoholic solution. Change of pH has no significance in the staining potential of the aqueous hibiscus solution when compared with the alcoholic hibiscus solution which increases in pH increases the staining potential partly. The concentration of the Hibiscus solution was observed to be significant as 5% hibiscus solutions show partly staining capabilities when compared with the 10% hibiscus solutions (Table 2) when compared with the control (Figs. 1 and 2).

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 colour, ++; Moderate colour, +++; Deep colour

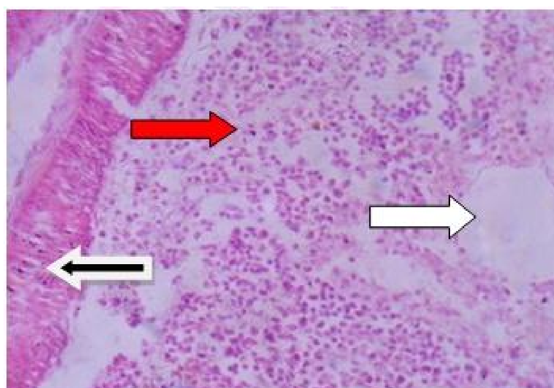

Fig. 1. Conventional Gram-stained lung tissue as control (X400)
Black arrow = Showing bacteria colony, Red arrow = Showing interstitial tissue, White arrow = Showing alveolar air spaces

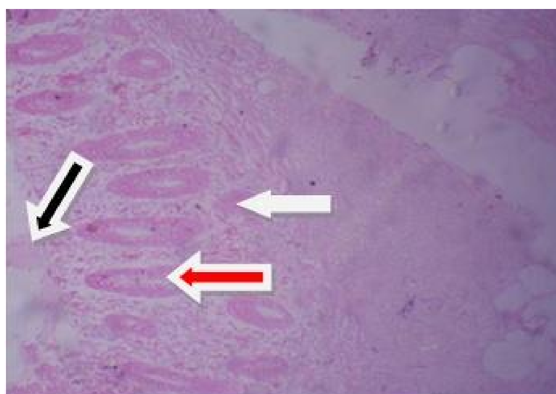

Fig. 2. Conventional gram-stained appendix as control (x100)
Black arrow = Showing intestinal lumen, Red arrow = Showing intestinal crypt, White arrow = Showing mucosal layer

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

Solvent	Concentration	Treatment	Time	Appendix		Lungs	
				Grade	Score	Grade	Score
	Control			++++	4	++++	4
Aqeous	5%	Untreated	30 seconds	+	1	+	1
			60 minutes	+	1	+	1
		AMH	30 seconds	++	2	++	2
			60 minutes	+	1*	++	2*
		GAA	30 seconds	++	2	++	2
			60 minutes	+	1	+	1
	10%	Untreated	30 seconds	+	1	+	1
			60 minutes	+	1	+	1
		AMH	30 seconds	-	0	-	0
			60 minutes	-	0	-	0
		GAA	30 seconds	+	1	+	1
			60 minutes	+	1	+	1
Alcohol	5%	Untreated	30 seconds	-	0	-	0
			60 minutes	-	0	-	0
		AMH	30 seconds	++	2*	+	1*
			60 minutes	+	1	+	1
		GAA	30 seconds	-	0*	+	1*
			60 minutes	+	1	+	1
	10%	Untreated	30 seconds	-	0	-	0
			60 minutes	-	0	-	0
		AMH	30 seconds	-	0	-	0
			60 minutes	-	0	-	0
		GAA	30 seconds	-	0	-	0
			60 minutes	-	0	-	0

*-; Undefined and unstained, +; Undefined and stained, ++; Define and stained, +++; Well defined and stained, ++++; Very well defined and well stained, *; Non corresponding, GAA; Glacial acetic acid, AMH; Ammonium Hydroxide*

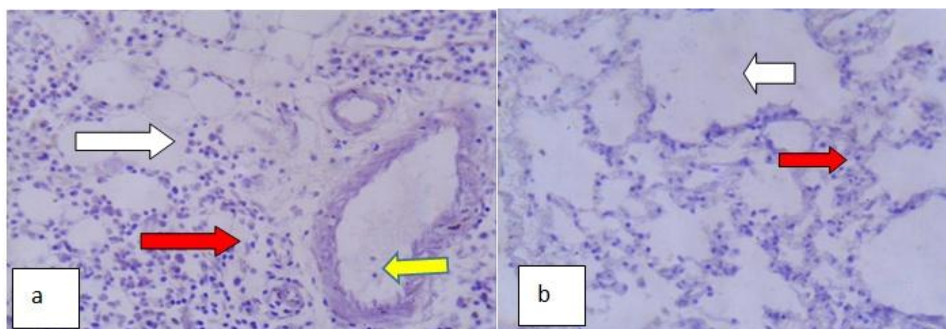

Fig. 3. (a) 5% aqueous H.S. ammonium hydroxide treated extract used to stain bacteria in lung tissue for 60 minutes. (b) 5% aqueous H.S. glacial acetic acid-treated extract used to stain bacteria in lung tissue for 30 seconds

Red arrow = Showing interstitial tissue, White arrow = Showing alveolar air spaces, Yellow arrow = Inflammatory cells

According to the result established, stains applied for a shorter duration have more staining impact when compared with the longer duration. 5% aqueous ammonium hydroxide treated extract at 60 minutes (Fig. 3a), 5% alcohol ammonium hydroxide treated extract and 5% alcohol glacial acetic acid-treated extract both at 30 seconds for bacterial staining gave non-corresponding result i.e. the intensity of the stains on the stained histological sections differs (Table 2) when the tissue elements (nuclei and cytoplasm) stained were compared between the lung and appendectomy tissue.

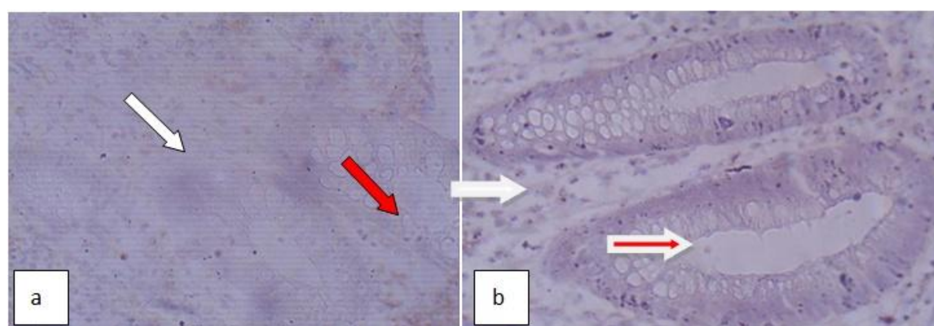

Fig. 4. (a) 10% aqueous h.s. extract used to stain appendicitis tissue for 30 seconds. (b) 10% aqueous h.s. glacial acetic acid-treated extract used to stain appendicitis tissue for 60 minutes (x400)

Red arrow = Showing intestinal crypt, White arrow = Showing mucosal surface

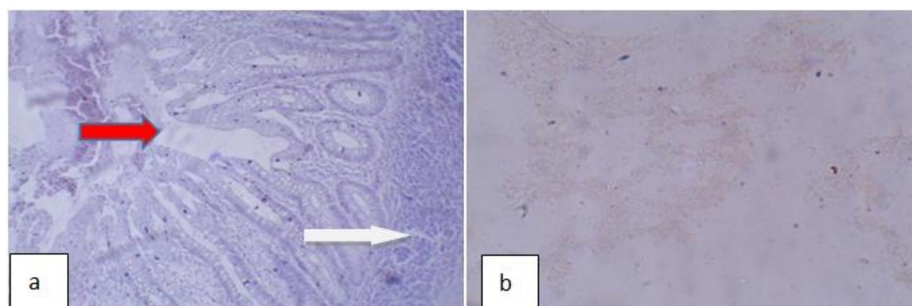

Fig. 5. (a) 5% alcoholic h.s. ammonium hydroxide treated extract used to stain appendicitis tissue for 60 minutes. (x100). (b) 10% of alcoholic h.s. extract used to stain bacteria in lung tissue for 60 minutes (x400)

Red arrow = Showing intestinal lumen, White arrow = Showing mucosal layer

4. DISCUSSION

In line with 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, used as a fungal and bacterial stain. In this study, it was observed that the *Hibiscus sabdariffa* powder have high solubility in alcohol than in boiled water which is not different with the result gotten in the research of Omorodion and Achukwu [12].

After changing the pH of the extracts in this work using a high quantity of ammonium hydroxide and glacial acetic acid different from the quantity used by Braide et al. [1] to demonstrate fungi and bacteria using different extracts, 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 research of Hashim [13], Ihuma et al. [14], Cheng et al. [15] and Chinyere and Etoforinini [16]. While in a work by Deepali [17], Hydrochloric acid was used to acidify the extracts in staining fungi and paramecium different from the approach used in this research and Braide et al. [1] research work.

In this study, different concentrations (5% and 10%) were used. The result obtained for the staining showed that 5% aqueous treated extract, 5% aqueous glacial acetic treated and 5% alcoholic ammonium hydroxide treated extract at shorter durations respectively, show better-staining effect on the tissue element which technically signifies that 5% concentration have significant staining ability than 10% concentration with the tissue elements stained blue in a faint-brown background, which support the work of Ibnouf et al. [12] and Raheem et al. [18], though their works were on tissue structure and not microorganism and also work of Braide et al. [1].

The bacteria were not well demonstrated by any of the solutions, which is in line with research work [5] that is on smear and this might be disputed due to the dark-blue stained nuclei of

the lungs and appendix tissues were intensified after counterstaining with *Hibiscus sabdariffa* solutions when compare with the control sections, which it might be as a result of the characteristics of the Bacteria (*Staphylococci*spp) of interest is a gram-positive (stains blue) bacteria or as a result of the affinity of the *Hibiscus sabdariffa* extracts with the nuclei staining them dark-violet in colour as reported by Ola et al. [11] and Benard [19].

The change of pH has no relative changes on bacteria but the background which is in line with Braide et al. [1] research work. It was observed in this work that staining duration at shorter duration gave better staining than the longer duration when compared with the control [14].

Based on the type of solvent used in this work, the aqueous extracts gave a better bacteria staining ability than the alcoholic extracts. Though couldn't demonstrate a definitive bacteria in the section, these results are concomitant with work done by Braide et al. [1] and Braide et al. [17].

Also, it was observed in bacterial staining that 5% ammonium treated aqueous at an hour, 5% ammonium treated alcoholic extract at 30 seconds and 5% G.A.A. treated alcoholic extracts at 30 seconds extracts did not give a corresponding grade of tissue elements stained when both lungs and the appendix were compared while others solutions showed same intensity grade and score, which dispute the work of Ibnouf et al. [12] and Raheem et al. [18] which may (be) due to difference in reaction of the cellular elements with the extracts or difference in modification of extracts [14].

The limitation observed is that none of the hibiscus solutions gave definitive resolution between the tissue cellular elements and the bacteria and the best results were obtained at longer duration, but inflammatory cells demonstration was established in lung tissue stained with 5% aqueous H.S. ammonium hydroxide treated extract at longer duration.

5. CONCLUSION

The *Hibiscus sabdariffa* extracts showed staining capability on tissue elements at shorter duration and demonstrated inflammatory cells than bacteria. Technically, it also signifies that 5% concentration has significant staining ability than 10% concentration with the tissue elements stained blue in a faint-brown background. Also, aqueous hibiscus solution showed stronger staining potential than the alcohol hibiscus solution. Manipulation of pH indicated no significance on the staining potentials. The results obtained signify that *Hibiscus sabdariffa* solutions could be used in diagnostic bacteriology.

ETHICAL APPROVAL

As per international standard or university standard ethical approval has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/55046