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The Use of Plant Dyes for Microbial Staining and Identification: An Eco-friendly and Non-Toxic Alternative Method

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Authors' contributions

This work was carried out in collaboration between all authors. Author SMA designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors AJA and GBA managed the analyses and were involved in the laboratory procedures of the study. Author GBA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Staining of microbial cells is a very important procedure in microbial identification. Cells need to be fixed and stained to increase visibility, accentuate morphological features and for preservation. The study was carried out to explore the efficiency of natural dyes from four different plants which can be used to stain bacterial cells.

Plant extracts which has been processed into dyes namely *Enantia chlorantha, Harungana madagascariensis, Sphenocentrum jollyanum* and *Sarcocephalus latifolius* were obtained from Botany Department, Obafemi Awolowo University, Nigeria. They were used to stain some Gram positive and negative bacterial cells. The dyes were extracted with Soxhlet apparatus using ethanol as solvent.

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The physiological features observed were compared with features of microorganisms that were stained with synthetic dyes when viewed under Olympus microscope at X100.
The dye from *Harungana madagascariensis* imparted its dark brown colour on the microbial cells within few seconds of application. A dark yellowish-brown dye from *Sarcocephalus latifolius* imparted a pinkish coloration on Gram negative organisms. Dye from *Enantia chlorantha* imparted light brownish colouration on Gram positive and light pink on Gram negative organisms. The dark yellow dye from *Sphenocentrum jollyanum* bark imparted greenish brown colouration on both Gram positive and light brown on Gram negative organisms. The best of the four dyes, *Enantia chlorantha* exerted a high fastness property which was seen when the slides were viewed.
The dyes from the plants when oxidized could be used as a suitable substitute for staining microorganisms. The procedure is simple, cheap, readily available and can be prepared easily. The applications and use of these natural dyes are found to be biodegradable, non-toxic and eco-friendly.

Keywords: Enantia chlorantha; Harungana madagascariensis; Sarcocephalus latifolius; Sphenocentrum jollyanum; microbial staining; natural dye; non-toxic.

1. INTRODUCTION

Dyes are substances of natural or synthetic origin, soluble in a medium which is usually used to impart a desired colour to a non-food material like paper, leather, wood, textiles and even cosmetics in a process known as dying [1]. Dyes are also referred to as stains and can be used to add colour to tissues, blood cells or organelles within individual cells as well as microorganisms such as bacteria, fungi and yeast to make them optically distinct [2].

The first use of dye in histology was credited to Antonie van Leeuwenhoek, the father of microbiology who worked with saffron, a natural dye extracted from *Saffron crocus* [3]. Stains are generally used to add colour to animal tissues, plant tissues, microbes and spores to make them optically distinct and the technique is known as Staining. 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 [4].

In Africa particularly in Nigeria, there are numerous natural dye plants which are capable of being cultivated, just as it has been cultivated in the India and United Kingdom. Recent studies have given useful result in which such abundant dye plants were used as histological stains for some tissue components [5]. These dyes are found in the root, root bark, leaves, flowers, stem, stem bark, fruit skins and nut shell [6]. The efficiency of some local natural herbal dyes for use in staining plant materials was found to be non-toxic and eco-friendly. They are also a source of cheap stains for use in plant histology [6].

Most stains in current use are chemically synthesized from cheap petroleum by-product, they show superior fastness property and wide variety of colours [7] and they are found to be hazardous to man's health [8,9]. Some synthetic dye components are carcinogenic or at least strongly allergenic resulting in their withdrawal as their hazard becomes recognized [10]. The usage of vast amount of synthetic dyes has resulted in water, land and air pollution which has greatly disturbed the earth ecological balance and cause health hazards [11]. As a result of this, manufacturing of synthetic dyes has been banned in some countries like India, Netherland and Germany which were the first producers of such dyes [8,12].

This has stimulated the search for alternative dyes for staining microbial cells, food samples, tissues and other materials which are relatively cheaper, eco-friendly and biodegradable. One of the ways to providing an alternative to synthetic dye is the provision of natural dye from plants and animal origin which has become an important topic due to the increase in environmental awareness targeted at reducing the deleterious effects of hazardous synthetic dyes to living things.

In this research, the staining potentials of four Nigerian plant extracts which include *Harungana madagasriensis, Enantia chlorantha, Sphenocentrum jollyanum, Sacrocephalus latifolius* were studied using selected bacteria. *Enantia chlorantha* Oliv (family-*Annonaceae*) common name-African Yellow Wood is widely distributed along the coasts of West and Central Africa and also very common in the forest regions of Nigeria [13]. The effectiveness of the extract was also compared with synthetic

staining reagents. These selected plants have been found to be of great importance due to their therapeutic abilities and safety for human consumption.

Several synthetic dyes (e.g. dyes with azo bonds nitro- or amino-groups) contain toxic heavy metals such as chrome, copper and zinc which are known to be carcinogenic; it also causes allergic-like symptoms [10]. Synthetic dyes have been implicated in being non- biodegradable and producing harmful waste to the environment which is toxic to man. The sources of synthetic dyes are non-renewable. The requirement of a professional in preparing synthetic dyes due to the specificity of accuracy in measurement has led to its unavailability when needed unlike natural dyes which are easy to prepare. Some synthetic dyes have been found to be highly inflammable which have resulted in fire outbreak in some laboratories and industries [9,12,10,14]. Therefore, there is a need for alternative source of dye which is easily available from plants which are eco-friendly, biodegradable, non-toxic to man and easy to produce.

The aim of this research is to provide additional information on the importance of using natural dyes as substitute for synthetic dyes in the identification and characterization of microorganisms. It will also enlighten microbiologists and pathologists on the effectiveness of some natural dyes when used to stain Gram positive or negative organisms, cells or tissues. It will explain the ease of using natural dyes to stain microorganisms.

2. MATERIALS AND METHODS

2.1 Extraction of the Natural Dyes

Dyes were extracted from *Harungana madagascariensis, Enantia chlorantha, Sphenocentrum jollyanum and Sarcocephalus latifolius* using Soxhlet appratus with ethanol as solvent [6].The crude dye extract can be used for tissue staining after extraction or can be further applied for solvent extraction in order to concentrate the dye solution before staining. Alternatively, the dried plant powder can be soaked to allow effective percolation and the powder can be extracted with solvent using Soxhlet Extractor (Steam Heated Extractor). The extract was concentrated using rotary evaporator and may further be dried by drying in the oven [5].

2.2 Extract Preparation

The concentrated extract in dried form was carefully picked using a spatula and weighed. Five grams of the dried extract was dissolved in 100% ethanol by continuous stirring until all the particles were completely dissolved. The dissolved extract was then sieved to remove tiny particles, after which it was poured into a clean covered bottle. A clean dropper was used in applying the dye on each slide.

2.3 Staining Procedure and Slide Preparation

The extracted dyes from the selected plant were tested on 18-24 hrs old bacterial culture which include *Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis*, *Lactobacillus plantarum, L. delbruckii, Aeromonas hydrophyla* . A loop was used to introduce distilled water to a grease free slide; the loop was sterilized by passing it through blue flame and was allowed to cool. The sterile loop was used to pick a distinct colony from the fresh culture and applied on the distilled water on the slide to make a thin film smear. The thin film smear was gently passed through the flame for one or two times and allowed to dry, after which a dropper was used to apply the dye on the slide and left to dry.

2.4 Microscopy

Microscopical observation of each slide was made using x100 objective lens. No immersion oil was used. The different features of the organisms were recorded and compared with standard staining technique (Gram's staining). Photomicrograph of each slides showing the microscopic features of each organism were taken using Olympus photomicroscope with analogue camera.

2.5 Gram's Staining

Gram's Staining was also carried out using the method of [15] as comparative study and analysis. This also served as control experiment.

3. RESULTS AND DISCUSSION

3.1 Results

The plates below show the photomicrograph of the cells of the different microorganisms when stained with different natural dyes and synthetic dyes and viewed under the microscope. These are shown in Plates 1-27.

Plate 1. *Staphylococcus saprophyticus* stained with *Harungana madagascariensis* x100

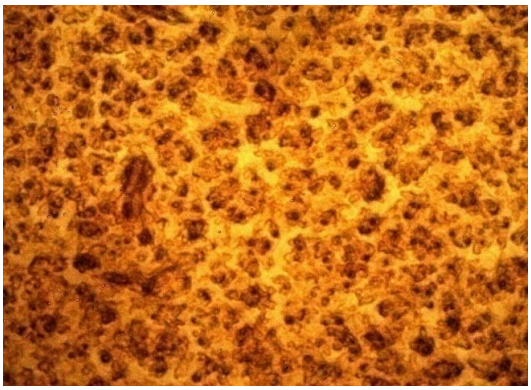

Plate 2. Staphylococcus saprophyticus stained with Gram stain

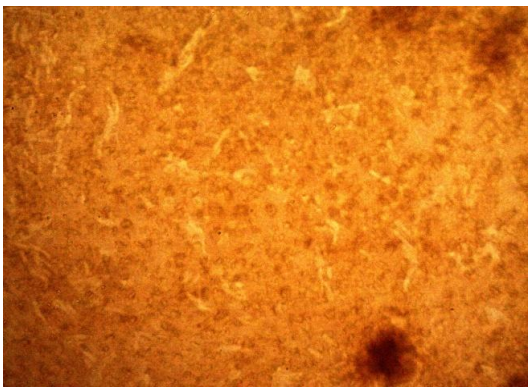

Plate 3. *Staphylococcus saprophyticus* stained with *Enantia chlorantha*

3.2 Discussion

Staining is an auxiliary technique used in microscopy to enhance contrast in the microscopic image. Stains and dyes are frequently used in biology and medicine to highlight structures in biological tissues for viewing, often with the aid of different microscopes [16,12,4].

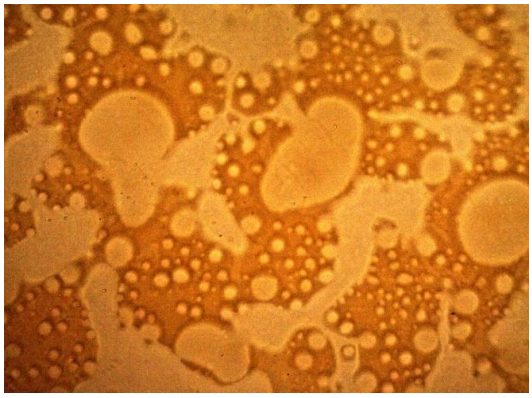

Plate 4. *Staphylococcus saprophyticus* stained with *Sphenocentrum jollyanum* x 100

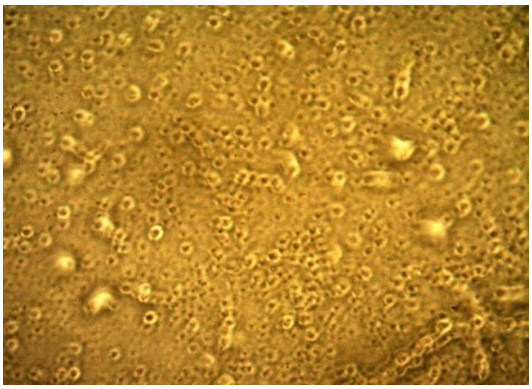

Plate 5. Staphylococcus saprophyticus stained with Sacrocephalus latifolius

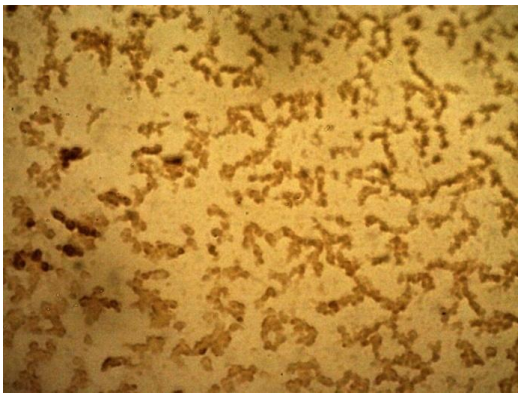

Plate 6. *Pseudomonas aeruginosa* stained with *Harungana madagascariensis* x100

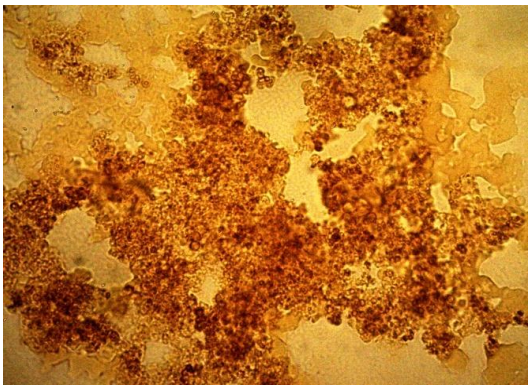

Plate 7. *Pseudomonas aeruginosa* stained with *Sarcrocephalus latifolius*

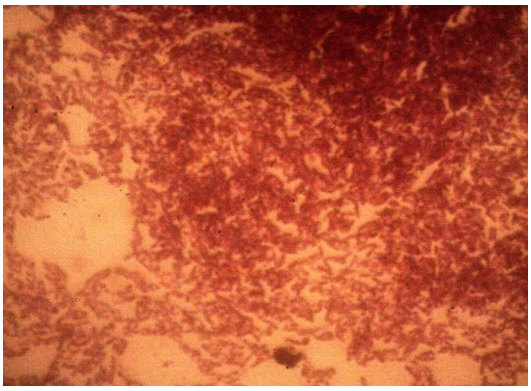

Plate 8. *Pseudomonas aeruginosa* stained with Gram's stain

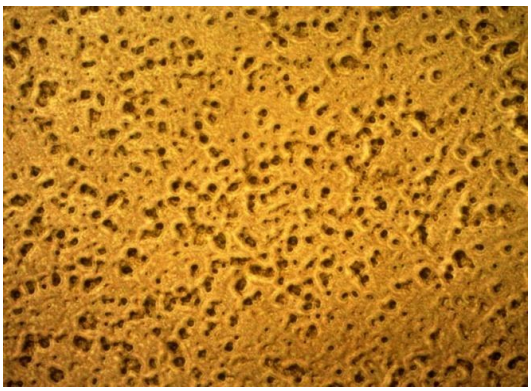

Plate 9. *Pseudomonas aeruginosa* stained with Sphenocentrum jollyanum x100

Stains may be used to define and examine bulk tissues (highlighting, for example, muscle fibers or connective tissues), cell populations (classifying different blood cells for instance), or organelles within individual cells [16,12]. In biochemistry, it involves adding a class-specific (DNA, proteins, lipids, carbohydrates) dye to a substrate to qualify or quantify the presence of a specific compound. Staining and fluorescent tagging can serve similar purposes. Biological staining is also used to mark cells in flow cytometry, and to flag proteins or nucleic acids in gel electrophoresis [4,10].

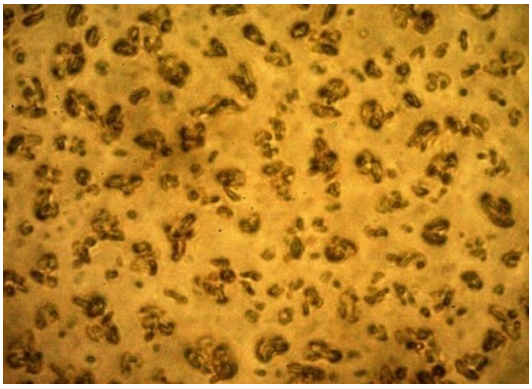

Plate 10. *Lactobacillus plantarum* stained with *Harungana madascariensis* x100

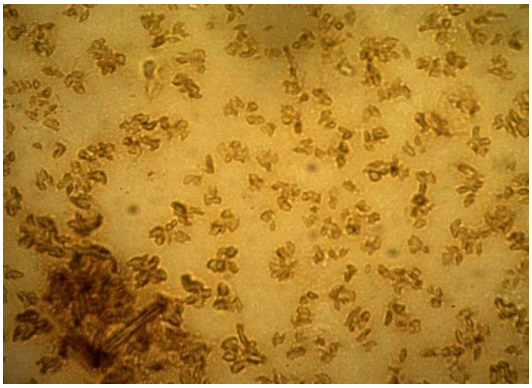

Plate 11. *Lactobacillus plantarum* stained with *Enantia chlorantha* x100

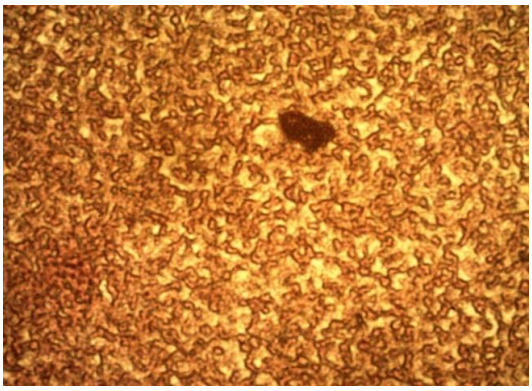

Plate 12. *Lactobacillus plantarum* stained with Gram's stain x100

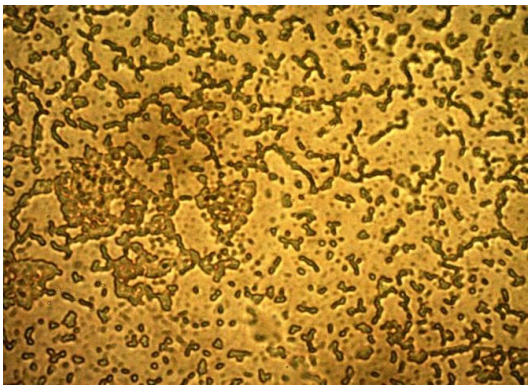

Plate 13. *Lactobacillus plantarum* stained with *Sphenocentrum jollyanum* x100

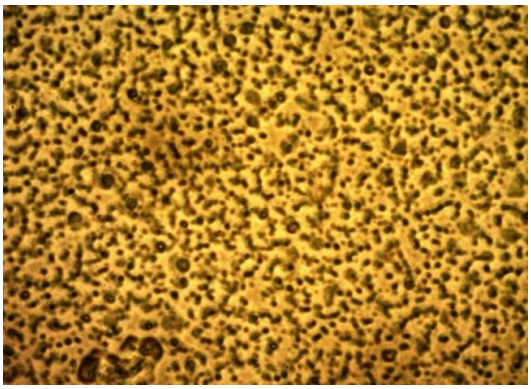

Plate 14. *Lactobacillus plantarum* stained with *Sarcrocephalus latifolius*x100

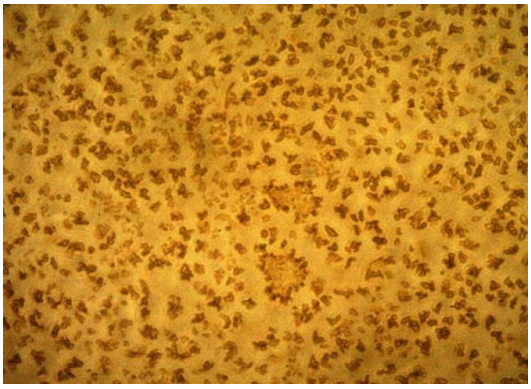

Plate 15. *Lactobacillus delbrueckii* stained with *Enantia chlorantha*

Simple staining is staining with only one stain/dye. There are various kinds of multiple staining; counterstaining, differential staining, or both, including double staining and triple staining. Staining is not limited to biological materials, it can also be used to study the morphology of other materials for example the lamellar structures of semi-crystalline polymers or the domain structures of block copolymers [2,4,10].

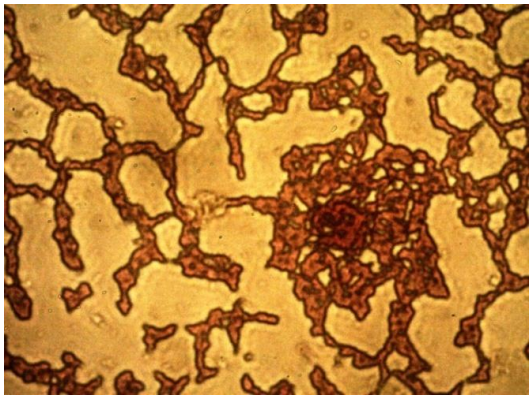

Plate 16. *Lactobacillus delbrueckii* stained with Gram's stain x100

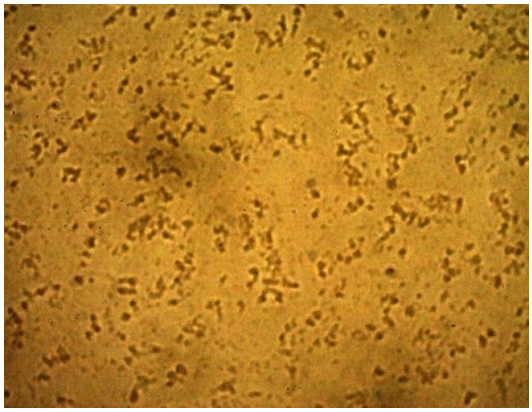

Plate17. *Lactobacillus delbrueckii* stained with *Harungana madascariensis* x10

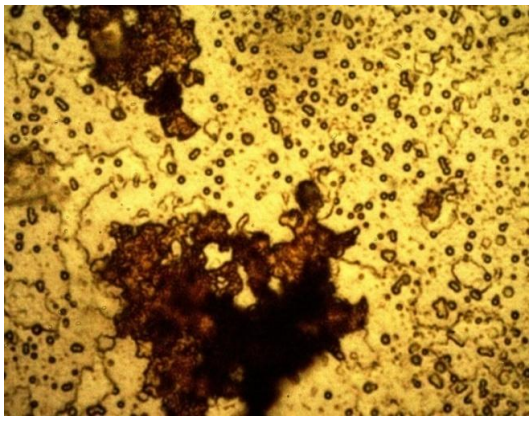

Plate 18. *Lactobacillus delbreukii* stained with *Sacrocephalus latifolius* x 100

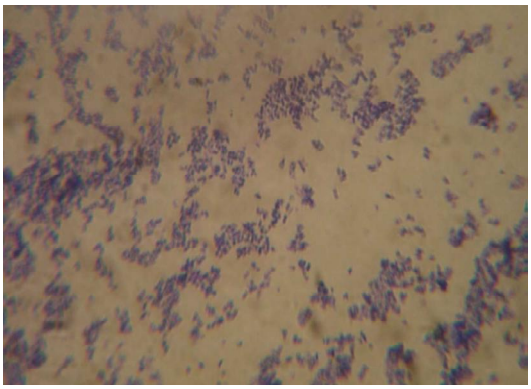

Plate 19. *Lactobacillus delbreukii* stained with Gram's stain

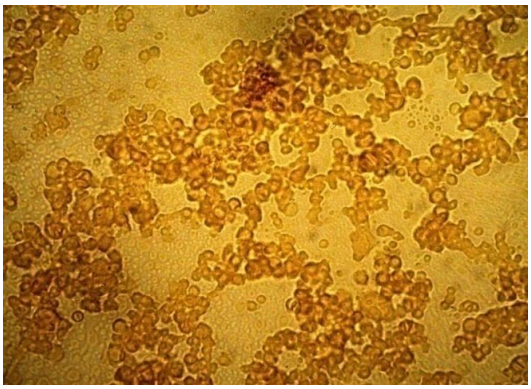

Plate 20. *Staphylococcus aureus* stained with *Sacrocephalus latifolius* x 100

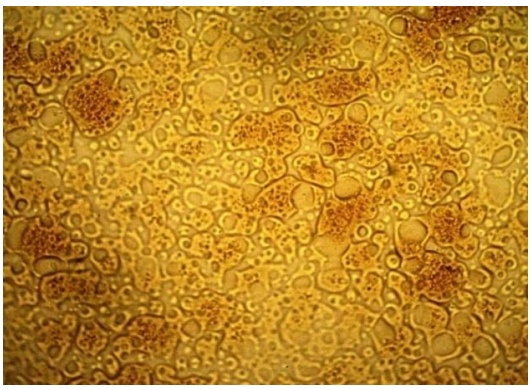

Plate 21. *Staphylococcus aureus* stained with *Sphenocentrum jollyanum* x 100

In this study, the cellular morphology of each isolates was seen clearly. A perfect distinction between the cocci and rod shaped cells were obtained. However, the colours obtained varied with the colour of each dye. Though generally, when compared with Gram's reaction, Gram positive cells stain darker than Gram negative cells. Gram negative cells appear lighter. This may be due to the presence or absence of teichoic acid and peptidoglycan layer of the different cell walls as reported by [4,3].

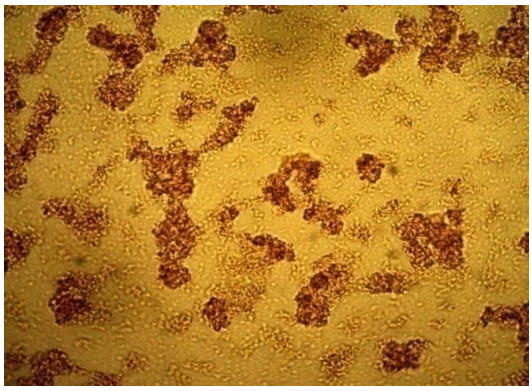

Plate 22. *Staphylococcus aureus* stained with *Harungana madagascariensis* x100

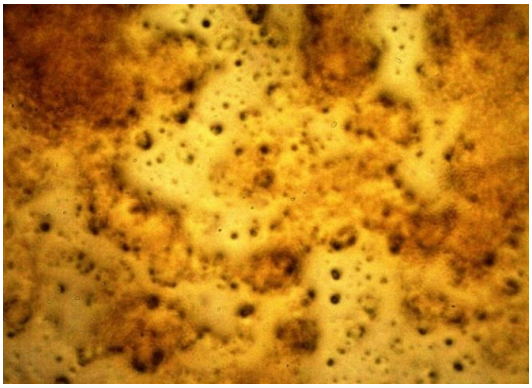

Plate 23. *Staphylococcus aureus* stained with *Enantia chlorantha*

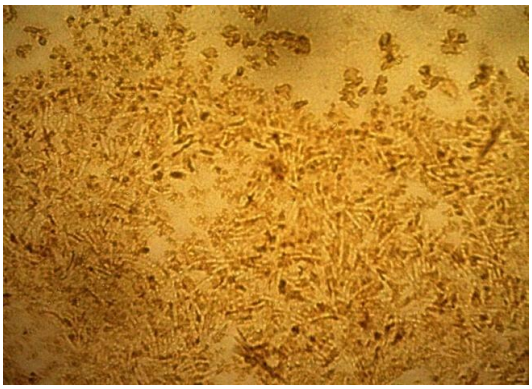

Plate 24. *Aeromonas hydrophila* stained with *Enantia chlorantha*

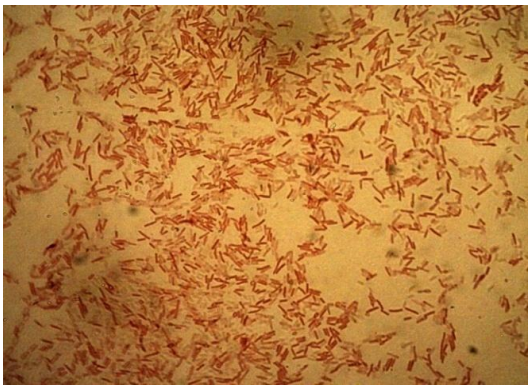

Plate 25. *Aeromonas hydrophila* stained with Gram's stain

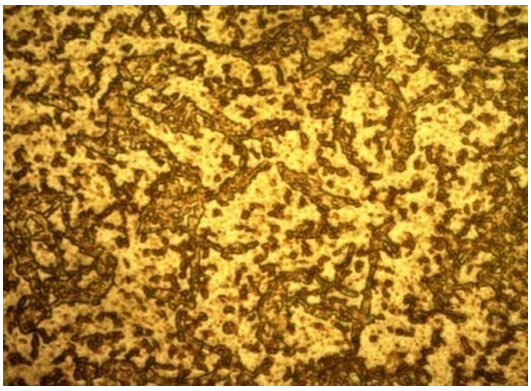

Plate 26. *Aeromonas hydrophila* stained with *Harungana madagscariensis*

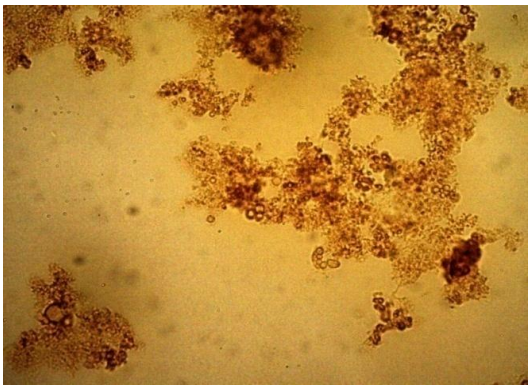

Plate 27. *Aeromonas hydrophila* stained with *Sacrocephalus latifolius*

However, with the use of plant dye, the stains were applied just once. There is no need for a mordant, decolourizer or a counter stain which makes the Gram staining reaction an elaborate process. This is corroborated by the work of [9,2]. The Gram's procedure may also be an elaborate and boring experience especially for young researchers because in microbial physiology, the quantity and timing of stains is very important. Accuracy of the procedure is also very important in each of the steps to be carried out.

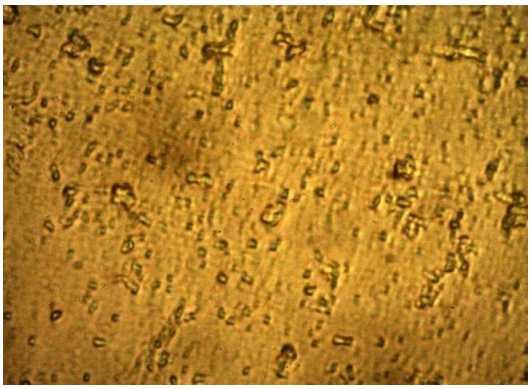

Plate 28: *Aeromonas hydrophila* stained with *Sphenocentrum jollyanum*

It is worthy of note to know that each of the stain have their own unique and characteristics colour which gives a better means of comparison between the four dyes. This also gives room for the comparison with the Gram's staining procedure which is based on the retention of the primary or secondary colours [1,7].

The dye extract from *Harungana madagascariensis* was crystallized and imparted its dark brown colour on the microbial cells within few seconds of application. Gram positive bacteria appears in shades of brownish colour. The organism appeared distinct when stained with dye extract from *Harungana madagascariensis.*

A dark yellowish-brown dye was obtained from *Sacrocephalus latifolius* plant; the extract crystallized within few minutes after application on microbial cells. This dye exerts a pinkish colour on Gram negative organism like *Pseudomonas aeruginosa* and appears with shades of brown on Gram positive organisms.

Dye extract from *Enantia chlorantha* imparts its light brownish /shades of yellow colouration on Gram positive organism and light yellow with brown tint on Gram negative as in the case of *Klebsiella pneumoniae*. It also crystallizes almost immediately when applied on a heat fixed smear. Microorganism appears distinct and clear when stained with extract from *Enantia chlorantha*.

A dark yellow dye was obtained from *Sphenocentrum jollyanum* stem bark which imparts greenish brown colouration on both Gram positive and a lighter green tint on Gram negative cells. Organisms do not really appear distinct when stained with this dye extract compared to the result from *Enantia chlorantha* dyes.

In addition, the cellular morphology of the different organisms using these different dyes were also distinct. One could see the way the cells were arranged either singly, in twos or in clusters as observed with the Gram's staining methods. This has also been observed by other authors [1,2,14]. Some of the plants have also been used in the past for staining plant histological samples [6], fungi [1,7], and staining of ova of intestinal parasites [5] and dyeing of clothes [8]. Some have also been used as either as primary stain, counter stain or mordant instead of the Gram staining reagents [2].

Some of the advantages of using natural dyes are; they are more eco-friendly than the synthetic dyes, as the synthetic dying procedure can produce pollutants and certain diazo-dyes which are carcinogenic. Most dyes are made from by-products of the petroleum industry and they may form recalcitrant by-products when they are not discarded properly [8,9,12,10,14].

Also, natural dyes are free from carcinogenic components; they are also known as antioxidants. In addition, depending on the mordant used, one dye can give variety of colours which depends on the source of the dye. Natural dyes are derived from natural sources unlike synthetic dyes; they are easily biodegradable and do not form recalcitrant by-products in the soil or in the environment [13,6,12,17].

Finally, the procedures are simple and easy to apply. You do not need an elaborate process to carry it out; it is simple and most natural dyes from plants are used once and they are washed off and viewed under the microscope. Students will therefore find the procedure very friendly and one that they can learn easily.

4. CONCLUSION

The dye extract from the four plants extract when oxidized, could be used as a suitable substitute for the usual stains use in Gram staining procedure in Nigeria and other countries where these plants are cultivated. Although the best of the four dyes is *Enantia chlorantha* because it has a high fastness property which could be seen when the slides were viewed. Also, the use of natural dyes in biological laboratories will facilitate the use of adequate protective measures for students and laboratory attendant; proper effluent treatment and disposal of natural and synthetic dyes.

Another advantage of this dye is that you do not need immersion oil to view your slides as in the case with the regular staining method. Once you stain, you can observe immediately after rinsing the dye off.

The applications of these natural dyes are found to be cheap, easy and more reliable. The extraction and application does not require specialized training. The dyes are also eco-friendly and biodegradable. With the increasing demand in the use of natural materials for environmental sustainability across the globe, these plants and the staining methods are highly recommended for use in microbiological and other laboratories.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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