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THE POTENTIALS OF HENNA (*LAWSONIA INAMIS* L.) LEAVES EXTRACTS AS COUNTER STAIN IN GRAM STAINING REACTION

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ABSTRACT
The potentials of aqueous and ethanol extract solutions of the henna plant (Lawsonia inamis L.) leaves adopted as alternative counter stain in Gram staining reactions were studied. Different extracts were formulated into various staining solutions of different concentrations. The solutions were modified with hydrogen peroxide, ferric chloride, potassium alum and potassium permanganate. These staining solutions were used to stain bacterial isolates of Lactobacillus spp and Escherichia coli strains. The experimental henna plant extracts solutions were used with usual counter stains (neutral red, safranin and dilute carbol fuchsin) as positive controls. The result obtained showed that aqueous extracts (cold and hot) oxidized with potassium permanganate with neutral pH gives a better staining reaction. While the ethanol extract after oxidized with potassium permanganate had no staining reaction. The result obtained therefore implied that the aqueous henna leaves extracts (cold or hot) when oxidized with potassium permanganate can be a substitute to the usual counter stains used in Gram staining reactions.
Keywords: Aqueous Extract, Dyes, Henna, Counter-Staining

INTRODUCTION

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. Stains may be used to define and examine bulk tissues (Highlighting, for instance, muscle fibers or connective tissues) cell populations, classifying different blood cells or organelles within individual cells (Penney *et al.,* 2002). A counter stain is a stain with color contrasting to the principal stain, making the stained structure more easily visible. These include the malachite green counter stain to the fuchsine stain in the Gimenez staining technique (Anonymous, 2011). Counter stain therefore is a stain that makes cells or structures more visible, when not completely visible with the principal stain. For instance crystal violet stain is applicable only to gram-positive bacteria in gram staining, while safranin counter stains are applicable to all cells allowing the identification of Gram-negative bacteria as well as Gram-positive strains. Often these stains are called vital stains. They are introduced to the organisms while the cells are still living. However, the stains are eventually toxic to the organisms. To achieve desired effects, the stains are used in very dilute solution ranging from 1:5000 to 1:500000. These stains may be used in both living and fixed cells (Horobin and Kiernan, 2002). But as the world cries for global economic crises, a resort to local and cheaper products is highly recommendable. And despite the elaborate and expensive medical technology today, the Gram's staining method remains an important and simple tool in medical bacteriology diagnosis. Therefore there is the need for innovation of new dye product that is simple and easy to handle and which might serve as counter stain in gram staining technique.

Henna (*Lawsonia inermis*) is a plant, which grow wild in abandoned areas (Muhammad and Mustapha, 1994). It is widely known as cosmetic agent used to color hair, skin and nails (Hanna *et al.,* 1998*)*. Henna has many traditional and commercial uses, the most common being as a dye for hair, skin and finger nails, as a dye and preservative for leather and cloth, and as an antifungal agent. Its flowers have been used to create perfume since ancient times (Bosoglu *et al*., 1998). It was used as a hair dye in India around 400CE (Auboyer, 1965), in Rome during Roman Empire and in Spain during Covivienca (Fletcher, 1992). Henna produces a red orange dye molecule, "*lawsone",* which is known as hennotannic acid, that has an affinity for bonding with protein and this has been used to dye skin, fingernails, hair, leather, silk and wool (Singh *et al*., 2005). But ironically, despite all these dyeing potentials of Henna extracts, they have not being reported to been use as biological stain especially as an alternative counter stain in Gram's staining reaction. This study therefore was aimed at employing the use of Henna leaves extract as counter stain of bacterial cell wall in Gram's staining reactions.

MATERIALS AND METHOD

The plant was obtained from a farm in Dass town, Dass local government area of Bauchi state, Nigeria. Identification of herbarium voucher specimen was carried out in the Federal Collage of Forestry Jos.

The plant has a voucher number: 900270. It is a small shrub of the family Lythraceae that produces dye in its leaves. The shrub, which is also called alkanna and mignonette tree grows in moist places. It bears small, fragrant, white or rose flowers in clusters. It is glabrous, multi-branched with spine tipped branchlets. Leaves are opposite, entire, glabrous, sub-sessile, elliptical and broadly lanceolate, acuminate, having depressed veins on the dorsal surface. Henna flowers have four sepals and a calyx tube with spread lobes. Petals are obvate, white or red stamens inserted in pairs on the rim of the calyx tube. Ovary is four celled. Fruits are small, brownish capsules, with 32–49 seeds per fruit and open irregularly into four splits (Kumar *et al*., 2005).

Preparation and Extraction Procedure

The leaves were dried, ground, and sieved. Three different extracts namely: Hot aqueous, cold aqueous and ethanol extracts were prepared as follows:

Hot Aqueous Extraction

Hot aqueous extraction was carried out according to the procedure described by Tandon and Rane (2008) and modified in Anonymous (2011). 50g of Henna leaves powder was weighed and soaked in 500ml boiled distilled water, shaken and left for 24hrs.The solution was filtered using No1 watt man filter paper. The filtrate was dried in a hot air oven at 30^{o}c for three days to get rid of residual water. The residue was scraped, ground, weighed and stored in a dried air tight container. The dried powder obtained was 9.03g with a pH of 4.03.

Cold Aqueous Extraction

Cold aqueous extraction was carried out according to the procedure described by Handa (2008). This was carried out as in an above except that cold distilled water was used instead of hot. The dried powder obtained was 10.10g and pH of 4.13.

Ethanol Extraction

Ethanol extraction was carried out according to the procedure described by Fatope *et al*. (1997). 50g of the powdered plant material was soaked in 300ml of absolute ethanol, macerated thrice and filtered; the filtrate was dried in a hot air oven for three days (Singh, 2008). The dried residue was scraped and the powder obtained was 4.98g with a pH of 2.86.

PREPARATION OF STAIN SOLUTIONS

From dry extract of the henna leaves obtained by aqueous and alcoholic extraction various staining solutions were prepared as follows: 2.5gbb of dried aqueous (hot and cold water extracts) was dissolved in 100ml distilled water in three different bottles each, one of the `solutions was ripened by oxidation with potassium permanganate, the other with hydrogen peroxide and the third solution was use without oxidation. Another 2.5g of dried extract was dissolved in 50ml distilled water in another two different bottles (to increase the concentration by 50%). One of the solutions saturated with potassium alum, the other with ferric chloride. More so, three different solutions of ethanol extracts were prepared by dissolving 1.25g of the extract in 50ml distilled water (the dissolved solution was filtered to remove some particles as it does not completely dissolved). One of solution of the solution was oxidized with potassium permanganate, the other with hydrogen peroxide and the third was use without oxidation.

Staining Procedure

Identified bacterial isolates of *Escherichia coli* and *Lactobacillus* were obtained from bacteriology laboratory of the Federal College of Medical Laboratory Technology NVRI Vom. Smears of bacterial colonies were made on clean grease-free slides which were clearly labeled, and Gram stained, using the different batches of Henna extracts as counter stains and controls were included, and the result was recorded.

RESULTS

The pH of varying solutions of henna extracts were determine using pH meter (Methrom Digital pH meter) and the results obtained are shown in Table 1. The result showed that the solutions at normal conditions are acidic but turned to near neutral by the addition of other substances.

Table 1 Identification of Hydrogen ion Concentration of Various Solutions of Henna Extracts

SOLUTION	pH VALUE
Hot aqueous extract without additional substances	4.03
Hot aqueous extract with potassium permanganate	7.16
Cold aqueous extract without additional substances	4.13
Cold aqueous with potassium permanganate	7.00
Ethanol extract without additional substances	2.86
Ethanol extract with potassium permanganate	6.55

The potential of an aqueous henna extract oxidized with Potassium permanganate as a counter stain on *Lactobacillus spp* was presented in Plate 1. The result showed that *L. spp* absorbed the henna stain and stained dark in the scenario as it occurs with the normal Sapranin stain (Plate 2). More so, the same result was found when *Escherichia coli* was counter stained with Sapranin (Plate 3) and compared with those counter stained with aqueous henna extracts (Plate 4).

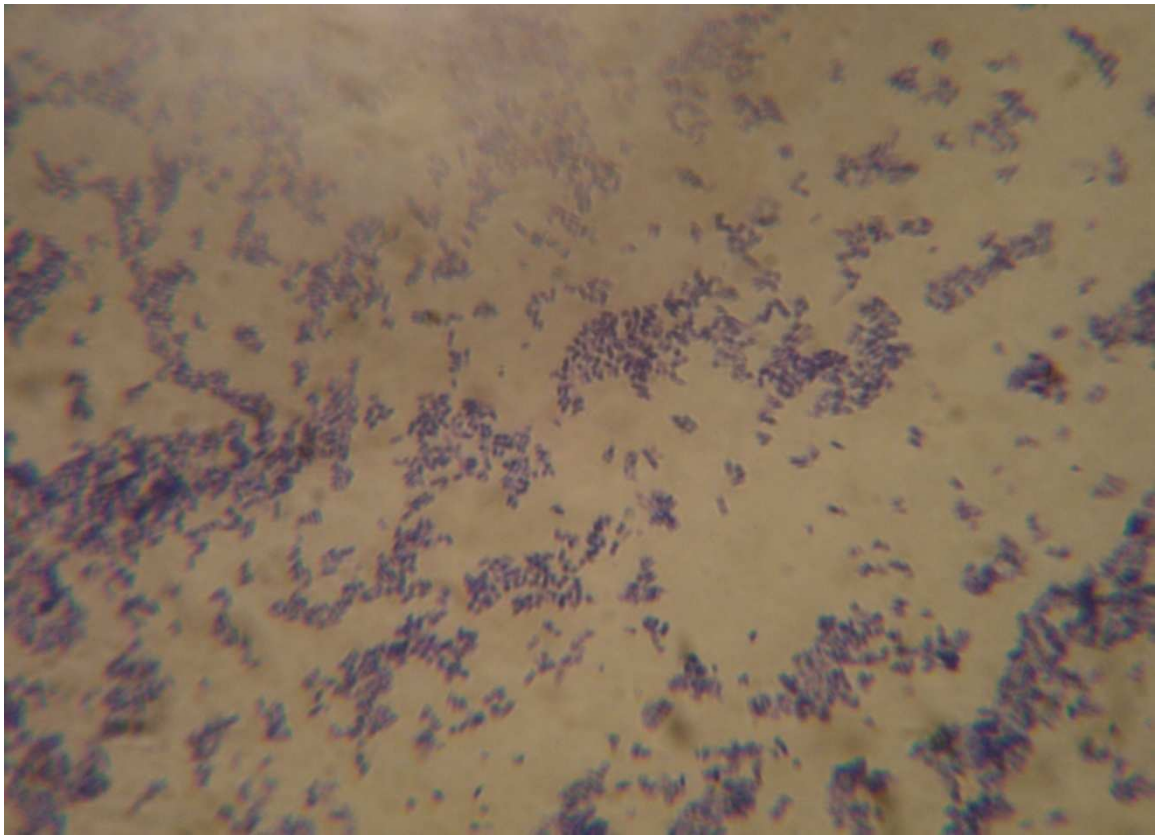

Plate 1: *Lactobacillus* counter stained with henna aqueous extract oxidized with potassium permanganate. (x100).

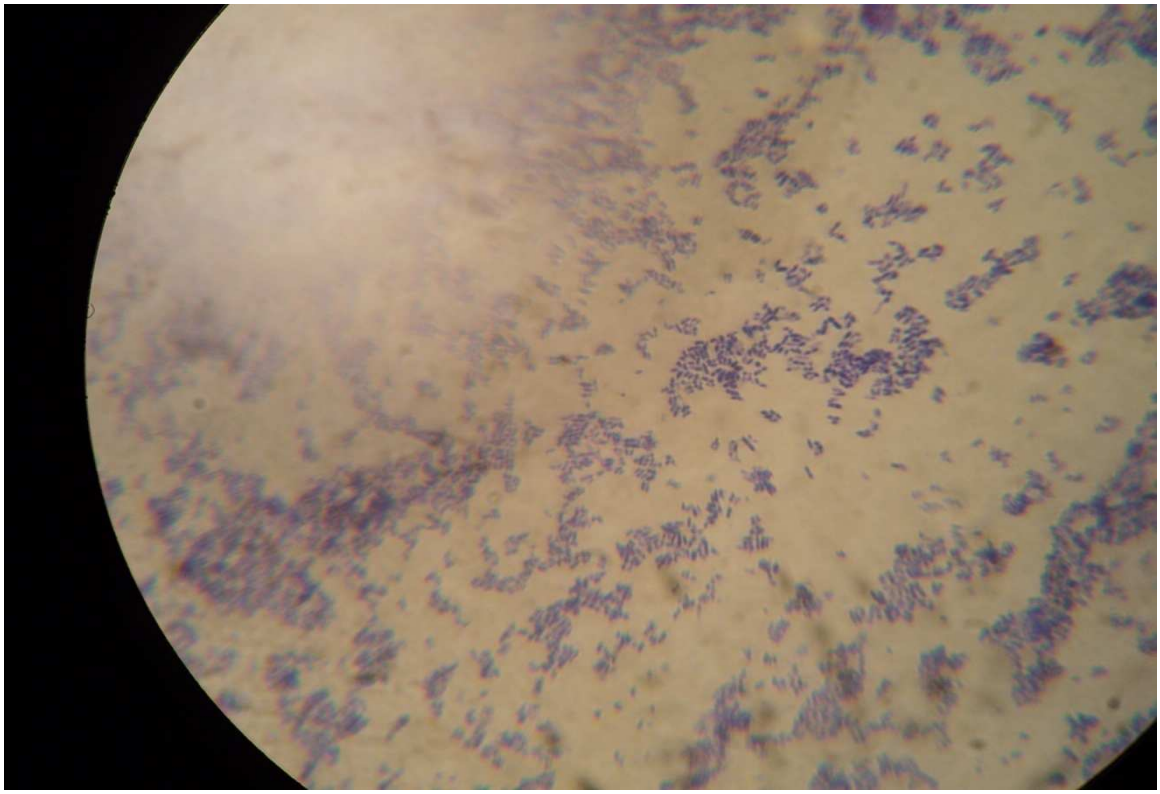

Plate 2: *Lactobacillus* counter stained with normal stain (safranin). (x100).

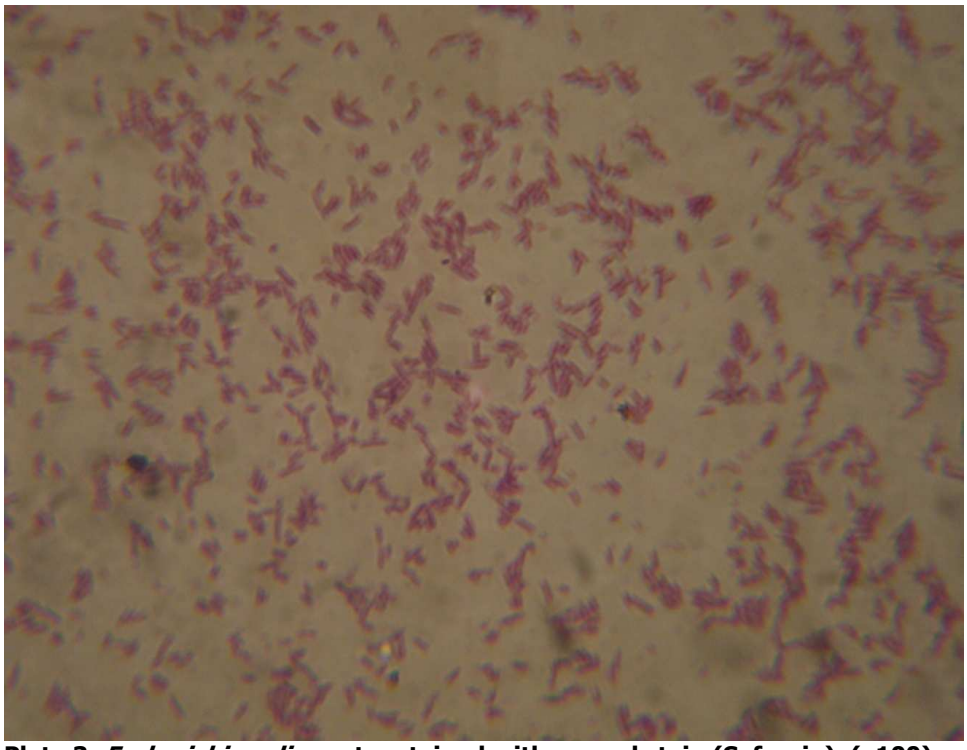

Plate 3: *Escherichia coli* counter stained with normal stain (Safranin). (x100).

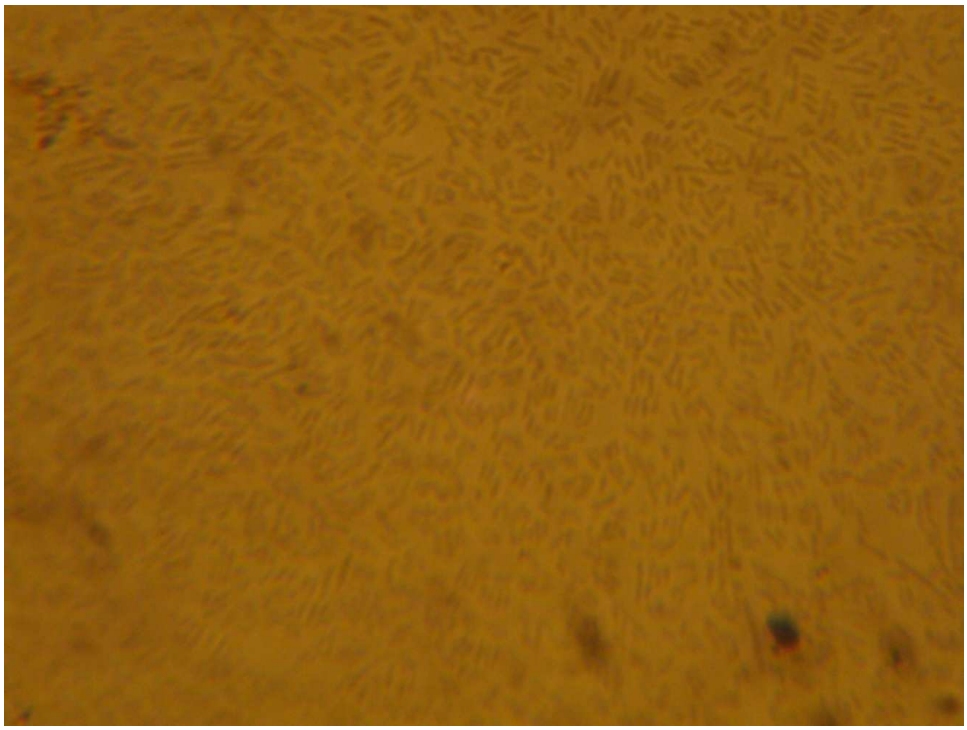

Plate 4: *Escherichia coli* counter stained with Henna aqueous extracts oxidized with potassium permanganate. (x100).

DISCUSSION

It is obvious that thirteen different Henna extracts stain solutions were used as counter stain in Gram staining the identified bacterial isolates, out of which only two (hot and cold extracts solutions both oxidized with potassium permanganate) stained *Escherichia coli* better, in shade of brownish color. However all the stain solutions when used as a counter stain on *Lactobacillus spp* do not present any difference with the positive control that was counter stained with normal stain (neutral red). This revealed that the henna aqueous (hot and cold) extracts oxidized with potassium permanganate impart a better staining reaction with *Lactobacillus spp* and *Escherichia coli* (Plate 1 and 4). This is in agreement with staining theory by Carleton (1996) that natural dyes need to berr ripened by oxidation either through natural means or by addition of chemical oxidants.

However other chemicals used in modifying the henna stain solutions either as oxidants or accentuators did not show staining reaction with gram negative bacteria. This may be attributed to their pH and color combination they produced when combined 22 henna extracts. It was observed that the aqueous extracts (both cold and hot) oxidized with potassium permanganate have a neutral pH as well as better color combination. This might be the reason why it gives a better staining reaction with *Lactobacillus spp*, because according to chemical staining theory the coloring matter of dyes is contained in the basic part of the compound, while the acidic radical is colorless and vice versa for acidic dyes as reported in Anonymous (2012). It therefore follows that acidic elements will have affinity for basic stains where as the basic structures have af3finity for acid stains as stressed by Ochei and Kolhatkar (2005). Therefore bacterial cells being rich in nucleic acid have a high affinity to basic dye, hence it was stained by the solution with neutral pH which is closer to basic pH and refused to pick the solutions with acidic pH. This is also evident from the pH of usual counter stains used in Gram stain technique which are highly basic.

In the case of *E. coli* used in this study all the henna extracts and reconstituted stains used as counter have shown proper staining reaction, as it retains the primary stain, without any alteration of color reaction as there was no difference between the bacteria counter stained with henna extract and that counter stained with normal counter stain. The ability of the bacteria to retain the primary stain after decolorization is attributed to nature of its cell wall which is thicker and have techoic acid and large amount of peptidoglycan layer which make a complex with mordant (Lugol's iodine) and resist decoloration as reported by Anonymous (2011). Henna extract, like most natural dyes contain tannins, saponins, alkaloids, resins, and glycoside. However in the context of staining, tannin (hennotannic acid or Lawsone) which is red colouring in the extract is the substances that make this plant a true natural dye. Saponins are known to reduce surface tension and this property enhances staining. In fact the various phytochemical constituents portray the henna plant extracts as a successful potential natural dye.

CONCLUSION

It was concluded that Henna leaves (cold or hot aqueous) extract when oxidized, could be used as a suitable substitute to the usual counter stains use in Gram staining procedure in Nigeria and other countries where henna plant is cultivated.

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