

Full Length Research Paper

Application of extracts of Henna (*Lawsonia inamis*) leaves as a counter stain

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Aqueous (cold and hot) and ethanol extracts solutions of the Henna plant (*Lawsonia inamis*) leaves was adapted for the first time as a counter stain in Gram staining reaction. Different extracts of *L. inamis* leaves were formulated into various staining solutions of different concentrations and modified with hydrogen peroxide, ferric chloride, potassium alum and potassium permanganate. These staining solutions were used to stain both known Gram positive and Gram negative bacterial isolates using Gram staining technique. The experimental Henna plant extracts solutions were used with usual counter stains (neutral red, safranin and dilute carbol fuchsin) as positive controls. Phytochemical screening of the extracts revealed the presence of tannin (hennatonic acid or Lawsone) and saponin. The aqueous extracts of the Henna plant (cold and hot) oxidized with potassium permanganate (pH 7.00 to 7.16) gave a better staining reaction with Gram negative bacteria, while the ethanol extract oxidized with potassium permanganate (pH 6.55) had no staining reaction with Gram negative bacteria. Hence 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 procedure. The results of staining ability of the various henna leaves extracts solutions are discussed.

Key words: *Lawsonia inamis*, extracts, bacteria, Gram staining, counter stain.

INTRODUCTION

A dye can generally be described as a coloured substance that has an affinity to the substrate to which it is being applied. The dye is generally applied in an aqueous solution, and may require a mordant to improve the fastness of the dye on the fibre. Dyes appear to be coloured because they absorb some wavelengths of light preferentially, and are obtained from animal and vegetable sources (Carleton et al., 1976). Until the middle of the nineteenth century, all dyes available to man came from natural sources, mainly vegetable extracts and a few from animal products. The range of colours was

limited as was the utility of dyes. The Gram's staining method is based on the ability of a cell to retain the crystal violet dye during solvent treatment, and on the difference in the microbial cell wall that is amplified. The cell walls for Gram-negative microorganisms have higher lipid content than Gram-positive cells (Bergey et al., 1994).

Henna, *Lawsonia inarmis* produces a red orange dye molecule, "lawsone", also known as hennotannic acid. This molecule has an affinity for bonding with protein and has been used to dye skin, fingernails, hair, leather, silk and wool (Singh et al., 2005). The leaves are also used as prophylactic against boils, burns, bruises, skin inflammations and as a gurgle against sore throat (Rout et al., 2001). The roots of this plant are useful in burning sensation, leprosy, strangury, premature grey of hair and

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tuberculostatic activity (Sharma, 1990; Vaidyaratnam, 1995). The major phytochemical constituents of Henna Lawson, is found to possess significant anti inflammatory, analgesic and antipyretic activities (Ali et al., 1995). Recently, this compound has been reported to have a growth inhibitory effect against human colon carcinoma, HCT- 15 cells (Kamei et al., 1998). Henna (*L. inermis*) is a plant, which grows wild in abandoned areas (Muhammad and Mustapha, 1994) and commonly known as 'Lalle' in Nigerian major languages with slight difference in pronunciation. This plant is a worldwide known cosmetic agent used to colour hair, skin and nails (Hanna et al., 1998).

The first human-made (synthetic) organic dye, mauveine, was discovered by William Henry Perkin in 1856. Many thousands of synthetic dyes have since been prepared. Synthetic dyes quickly replaced the traditional natural dyes. They cost less, offer a vast range of new colours, and impart better properties upon the dyed materials (Rosenberg, 1971).

Despite the biotechnological advance in medical science today, biological stains are vital in laboratory diagnosis and the Gram's staining method remains an important simple diagnostic tool in diagnostic and research laboratories. The commonly used counter stain in Gram's staining reaction is safranin. Safranin is also used as counter stain in endospore and capsule staining. Acid fast staining used in identifying all bacteria in the Genus *Mycobacterium* including the two important disease producers *Mycobacterium tuberculosis* and *Mycobacterium leprae* uses carbol fuchsin as primary stain and methyl blue as counter stain. Today dyes are synthetically prepared from aromatic compounds like coal tars the derivative of natural dyes Henna, (*L. inermis*) that are available and can easily be processed. Despite the benefits of Henna, *L. inermis*, it has not been reported as a biological stain. In this study, we report the use of Henna, *L. inermis* extracts as a natural counter stain in Gram's staining reaction.

MATERIALS AND METHODS

Experimental organisms

The clinical isolates of *Escherichia coli* ATCC25922, *Listeria monocytogenes* ATCC15313 ATCC27853 and *Staphylococcus aureus* ATCC28923 used were obtained from Molecular Biology Department, Federal College of Veterinary and Medical Laboratory Technology, National Veterinary Research Institute, Vom, Nigeria.

Plant materials

Fresh leaves of Henna (*L. inermis*) identified by a taxonomist in the Federal College of Forestry Jos, Nigeria, were collected from a farm in Dass town of Dass Local Government Area, Bauchi State, Nigeria. The fresh leaves were air dried for three weeks in the molecular biology laboratory at the Department of Molecular Biology, Federal College of Veterinary and Medical Laboratory

Technology, Vom. The dried leaves were grounded using pestle and mortar and carefully packed in sterile polythene bags weighed and stored under room temperature until used (Figure 1a and b).

Preparation of crude extracts from the powdered leaves of *L. inermis*

Hot aqueous extraction

Two hundred grams of the powdered leaf of *L. inermis* was soaked in 1000 ml boiled distilled water, shaken and left for 24 h. The solution was filtered using No.1 watt man filter paper. The filtrate was dried in a hot air oven at 30°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 9.03 g (pH 4.03) (Harbourne, 1998).

Cold aqueous extraction

This was carried out with the method above except that cold distilled water was used instead of hot. The dried powder obtained was 10.10 g with pH of 4.13.

Ethanolic extraction

200 g of powdered *L. inermis* were packed into a thimble and transferred into Soxhlet extractor with 1 L of 95% ethanol for about 72 h until there was no colour change in the ethanol, indicating the end point of extraction completion. The extract was harvested and concentrated in a rotary evaporator separating the ethanol from the real extract. However, the remnant ethanol in the extract was removed by placing the extract in porcelain dishes in the oven at 80°C until the weight remained constant. The extract weighed 4.98 g (pH 2.86) and was then collected in air tight plastic container and stored in the refrigerator at 4°C ready for use.

Phytochemical screening

The extracts of *L. inermis* were screened for phytochemical constituents according to Trease and Evans (1983) and Harbourne (1998).

Preparation of extract solution of *L. inermis* for Gram reaction

Aqueous extracts

2.5 g of dried aqueous (hot and cold water extracts) was dissolved in 100 ml of distilled water in three different bottles each, one of the solution was ripened by oxidation with potassium permanganate, the other with hydrogen peroxide and the third solution was used without oxidation. Solution A and B was ripened with potassium permanganate and hydrogen peroxide, while solution C was used without oxidation. Another 2.5 g of dried extract was dissolved in 50 ml 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.

Alcoholic extract

Three different solutions of ethanol extracts were prepared by



a



b

Figure 1. (a) Henna plant, (b) Henna powder.

dissolving 1.25 g of the extract in 50 ml distilled water (the dissolved solution was filtered to remove some particles as it does not completely dissolve). One of the solutions was oxidised with potassium permanganate, the other with hydrogen peroxide and the third was used without oxidation. Another 2.5 g of dried extract was dissolved in 50 ml 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. Hydrogen ion (pH) concentrations of various Henna extracts were determined using pH meter (Methrom Digital pH meter) and recorded.

Hot aqueous extract without additional substances; 4.03, hot aqueous extract with potassium permanganate; 7.16, hot aqueous extract with hydrogen peroxide; 3.41, hot aqueous extract saturated with potassium alum; 2.72, hot aqueous extract saturated with ferric chloride 1.02, cold aqueous extract without additional substances; 4.13, cold aqueous with potassium permanganate; 7.00, cold aqueous with hydrogen peroxide; 3.40, cold aqueous extract saturated with potassium alum; 2.72, cold aqueous extract saturated with ferric chloride 1.02, ethanolic extract without additional substances; 2.86, ethanol extract with potassium permanganate 6.55 and ethanolic extract with hydrogen peroxide 2.01.

Staining organisms with henna extract

Smears of the *E. coli* ATCC25922, *L. monocytogenes* ATCC15313 and *S. aureus* ATCC2892 were made and fixed. These smears were Gram stained with Gram's reagents and counter stained along with the different batches of Henna extracts as counter stains. In every batch one positive control (counter stained with normal

counter stain) and one negative control (with no counter stain) were included, and the result was recorded.

RESULTS

Phytochemical analysis

The extracts of *L. inermis* gave positive tests for alkaloids, saponin, tannin and glycosides and negative for flavonoids and resin.

Staining reaction of henna extract on experimental bacteria

Thirteen differently constituted Henna extract stain solutions were used as counter stain in Gram staining two identified Gram positive and two identified Gram negative bacteria, out of which only two (hot and cold extracts solutions both oxidised with potassium permanganate) stain Gram negative bacterium (*E. coli*) better, in shade of brownish colour.

However, all the stain solutions when used as a counter stain on Gram positive bacteria did not show much difference with the positive control that was *Staphylococcus* and *L. monocytogenes* counter stained



Figure 2. Oxford *S. aureus* ATCC 28923, counter stained with aqueous henna extract X100.

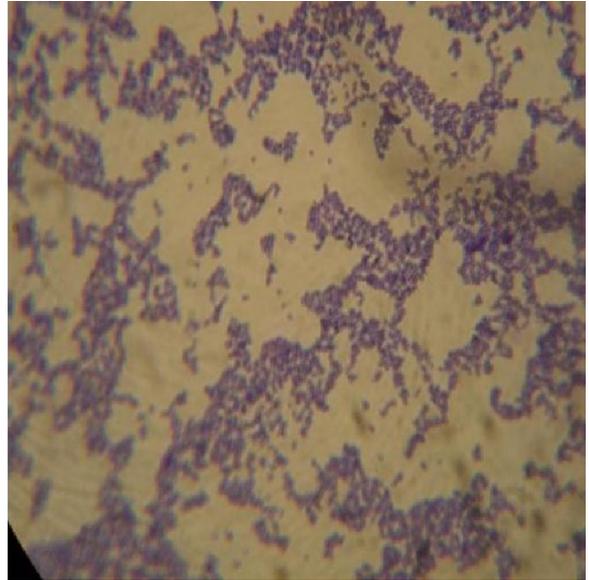


Figure 3. Oxford *S. aureus* ATCC 28923, counter stained with neutral red X100.

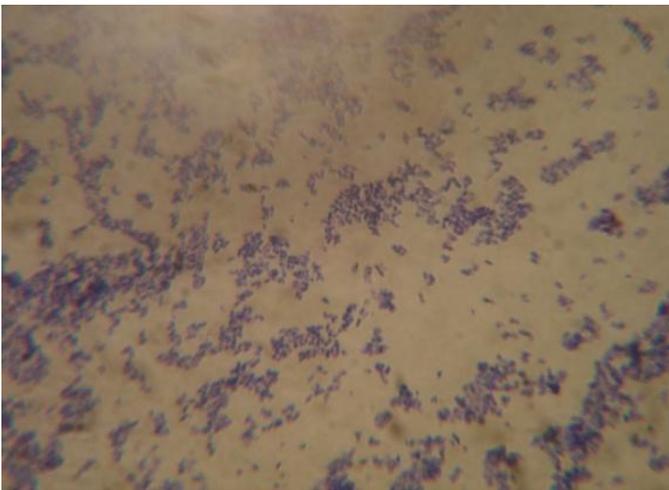


Figure 4. *L. monocytogenes* ATCC15313 counter stained with henna extract X100.

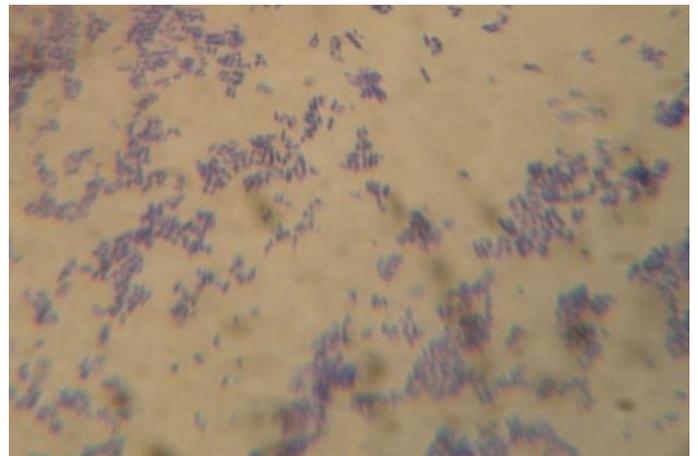


Figure 5. *L. monocytogenes* ATCC15313 counter stained with safranin X100.

with normal stain (neutral red). The Henna extracts stain solution and the conventional counter reactions on the experimental bacteria are shown on Figures 2 to 7.

DISCUSSION

From the experimental results, it was observed that the Henna aqueous (hot and cold) extracts oxidized with potassium permanganate impact a better staining reaction with Gram negative bacteria, after an attempt to

use the extract alone without oxidation proved abortive. This no doubt may be associated with the staining theory of Carleton et al. (1976) that natural dyes need to be '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 colour combination produced when combined with henna extracts. For instance when ferric chloride was added it turned to black colour with a shift of pH from 4.03 to 1.02 which is highly

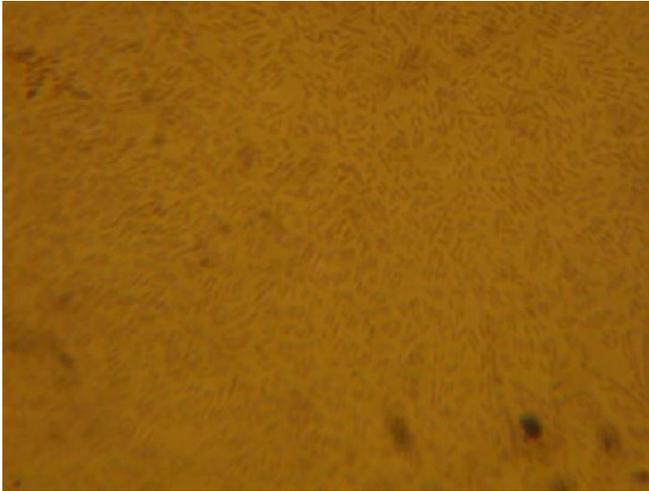


Figure 6. *E. coli* ATCC 25922 counter stained with henna aqueous extract X100.

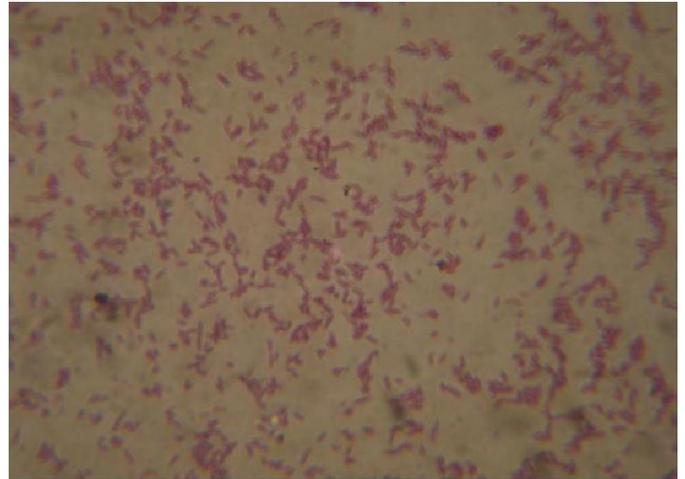


Figure 7. *E. coli* ATCC 25922 counter stained with Safranin, X100

acidic; with potassium alum it turned to dark greenish colour and pH shift to 2.72 which is also very acidic; when combined with hydrogen peroxide the solution turned to light brown with pH of 3.40 which is also acidic. But by oxidation with potassium permanganate the solution became dark brown with the neutral pH 7.0.

It was observed that the aqueous extracts (both cold and hot) oxidized with potassium permanganate have a neutral pH as well as better colour combination. This might be the reason why it gives a better staining reaction with Gram negative bacteria, because according to chemical staining theory the colouring matter of dyes is contained in the basic part of the compound, while the acidic radical is colourless and vice versa for acidic dyes. It therefore follows that acidic elements will have affinity for basic stains whereas the basic structures have affinity for acid stains (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. The inability of ethanol extracts to stain the Gram negative bacteria may be attributed to the difficulty encountered in attempting to make aqueous solution of the extract, and since alcohol is known to remove stains from the cells it should not be employed.

In the case of Gram positive bacteria used in this study all henna extracts reconstituted stains used as counter has shown proper staining reaction, as the Gram positive bacteria retains the primary stain, without any alteration of colour reaction as there is no difference between the Gram positive bacteria counter stained with Henna extract and that counter stained with normal counter stain

(Figures 2, 3, 4 and 5). The ability of Gram positive bacteria to retain the primary stain after decolourization is attributed to the nature of its cell wall which is thicker than those of Gram negative and have teichoic acid and large amount of peptidoglycan layer which forms a complex with mordant (Lugol's iodine) and resist decolouration. 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 substance that makes 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

In conclusion the Henna leaves aqueous extract cold or hot when oxidised, could be used as a suitable substitute to the usual counter stains used in Gram staining procedure. Based on the findings in this study, we therefore, conclude that the oxidized extract of *L. inermis* has the potential of being employed as a counter stain in Gram's staining reaction.

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