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The use of *Aloe barbadensis M.* (Aloe vera) Extract as Potential Stains in Gram's Staining Technique

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Abstract
Aloe barbadensis miller (Aloe vera) aside being a potent antioxidant with effects attributed to the phytoconstituents (anthraquinone), can also serve as natural dyes. This study explored the staining potentials of A. barbadensis extract on microbial specimens in modified Gram's staining technique. The plant was washed; shrubby edges of the leaves were scraped off and each leaf cut open to expel the gel content. The dried and grinded leaf material weighing 450g was treated with two different extraction methods (150g for heated alcoholic aloe extract (HAAE) and 150g each for the unheated alcoholic aloe extracts I & II (UAAE-1 & UAAE-II). Preliminary phytochemical screening of extracts using bontragers test was conducted. Solutions from the two extraction methods with and without a mordant were applied on bacterial smears. Anthraquinone-rich extract under optimal extraction conditions were 10.0, 4.84 and 18.8 respectively. Comparing the extraction methods, HAAE and UAAE1 & UAAEII are mutually convenient and easy to make; but the former was more cost-effective with regards to power and instrumentation while the latter was more cost-efficient with regards to solvent. The pH of various prepared solutions was acidic and the extracts contained a bioactive agent that imparts pigment on biological specimens. In comparing the mordanted and non-mordanted solutions, the former had no additional effects on the staining efficiency of the aloe dye extract. We concluded that A. barbadensis dye visibly stained bacterial Gram positive and Gram negative cells in modified Gram's staining without structural differentiations. We recommend reductions in staining duration for microbial samples to ascertain the reasons for the variation in staining uptake.

Keywords: *Aloe barbadensis,* anthraquinone, dyes and stains, Gram staining

INTRODUCTION
Aloe barbadensis miller belongs to the family Xanthorrhoeaceae which was formerly identified as Liliaceae, Aloaceae or Asphodelaceae (Chen and Van-Wyk, 2012). The genus Aloe contains over 500 species which differ significantly in their appearance (heights and sizes) ranging from very small shrub-like plants to very large trees (Grace, 2011). However, two species; *A. barbadensis* and *A. aborescens* are used for trading world-wide (Sharrif and Sandeep, 2011). Aloe leaf can be divided into two main parts; the sticky latex liquid resulting from the yellowish green pericyclic tubules that line the outer rind; this is the part that yields laxative anthraquinones and the inner clear colorless fresh parenchymal containing the aloe gel

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(Schulz *et al.,* 1997). Chemical constituents of the leaves of *A. barbadensis* comprised of anthraquinones and anthrones from the middle layer; latex leaf lining and polysaccharides from the gel (Hamman, 2008; Surjushe *et al.,* 2008).

Available literatures suggested that due to the presence of secondary metabolites in Aloe plant, it possesses abundant biological properties (Boudreau *et al.,* 2013). Its numerous uses traditionally in cases of inflammation, burn injury, fever, eczema and cosmetics have also been recounted (Egbuna *et al.,* 2020). Conversely, there are lethal species of Aloe plants that result to adverse effects like hypokalemia, diarrhoea, kidney failure, hypersensitive reactions, phototoxicity or cytotoxicity and pseudomelanosis coli (Guo and Mei 2016).

To a large extent, *A. barbadensis* remains a promising plant as a multipurpose medical agent to which clinical trials could be done for novel drugs production (Egbuna *et al.,* 2020). A number of anthraquinones are found in leaves and roots of the species of aloe genus (Dagne *et al.,* 2000). Anthraquinone is a generally acceptable and important raw material for the production of a class of water-insoluble dyes (vat dyes); capable of imparting colors to blood cells or organelles within individual cells, microorganisms: fungi, bacteria and yeast, including tissue sections to aid visual and optical differentiations (Chukwu *et al.,* 2011). A number of synthetic dyes have been recognized as carcinogenic in addition to their capability to inflict bodily complications. Alternately, the effectiveness of several indigenously prepared herbal and natural dyes were found to be atoxic, ecofriendly and sustainable. It is against this backdrop, that the present study conceptualized the need for an unconventional dye source, easily ready for use and obtainable from plants not excluding its eco-friendliness demonstrated in gram staining technique.

Aloe multipurpose application has again prompted the exploration of alternative dyes intended for staining bacterial cells. It has a potential significance of breaking the monopolized existence of the neutral red or crystal violent, which are the main primary and counter stains in gram staining techniques. Hence, the staining potentials of *A. barbadensis* extract were studied on selected microbial isolates via the modified gram staining techniques.

MATERIALS AND METHODS

Study Area

Analysis and staining process with the *A. barbadensis* dye was carried out in the laboratory of the department of Medical Laboratory Science, University of Benin, Edo State.

Collection of Plant

A. barbadensis plant was obtained from a private plant garden in Benin city.

Plant Identification and Authentication

It was identified and authenticated by an expert taxonomist in University of Benin. A sample of the Aloe plant was deposited in the herbarium of the Department of plant Biology and Biotechnology University of Benin.

Ethics Clearance

Clearance for this study was obtained from the Faculty of Pharmacy, University of Benin (EC/FP/021/13).

Extraction of *A. barbadensis*
The leaves were washed while shrubby edges were scraped off by cutting each leaf open to remove the aloe gel content. The leaves were dried in a shaded environment for seven days and grinded to powder using an electric blender.
The fine particles were weighed using an electronic balance and treated with three different extraction methods to obtain the heated alcoholic aloe extract (HAAE), unheated alcoholic aloe extract I (UAAEI) and unheated alcoholic aloe extract II (UAAEII) respectively.

Heated Alcoholic Aloe Extract (HAAE)
Methanol (500ml) containing 10% ferric chloride and 5% hydrochloric acid were mixed thoroughly and added to 150g of the powdered Aloe leaf. It was placed on a heating mantle and stirred to ensure uniform heating. After boiling, it was allowed to cool and stirred thoroughly. The mix was left to stand for 24 hours before filtration using Whatman No.1 filter paper while the marc was re-extracted with fresh methanol (1000ml), and placed on a water bath over steam for methanol to evaporate. Water was later added to the concentrate and mixed while chloroform (2000ml) was added to the mixture and allowed to stand overnight. On separation after standing, the aqueous layer was left in the separation funnel while the layer of chloroform was flowed out. Then, on a boiling water bath, the crude extract was evaporated, lyophilized and weighed.

Unheated Alcoholic Aloe Extract 1 (UAAE1)
The grinded plant material was measured (150.0g), tied in a thimble and placed in a soxhlet apparatus. Methanol (1000ml) was used as the extraction solvent while extraction process continued until the solvent was clear. The extract was placed on a boiling water bath over steam to cause the methanol to vaporize. 100ml of water mixed with 10% ferric chloride and 5% hydrochloric acid ferric chloride was employed in the total anthraquinone isolation. The mixture was thereafter combined with the concentrate and mixed thoroughly while the rest of the procedure continued in line with the previous extraction method.

Unheated Alcoholic Aloe Extract II (UAAEII)
Grinded aloe leaf powder was measured (150.0g) while the extraction processes were in line with UAAEI except that ferric chloride and hydrochloric acid were excluded.

Identification of Anthraquinones
Phytochemical screening for anthraquinones was carried out as described by Harborne (1998). The presence of anthraquinone in test plant extracts was determined by Borntrager's test while absolute methanol and chloroform were used for extracting total anthraquinones rich extracts.

Bacterial Isolate from Culture Plates
Isolates of *Staphylococcus aureus, Escherichia coli* and *Klebsiella pneumoniae* were all isolates obtained appropriately from Microbiology unit, Department of Medical Laboratory Science University of Benin. Pure isolates cultured on the respective selective media were obtained. Biochemical tests (Catalase, Coagulase, Indole, Citrate and Oxidase) were performed to identify and confirm isolates. Bacteria Smears of organisms (*Staphylococcus aureus, Escherichia coli* and *Klebsiella pneumoniae*) were made on clean grease free slides and heat fixed.

Preparation of *A. barbadensis* Staining Solution
One gram (1g) of extract each (HAAE, UAAEI and UAAEII) was dissolved in 100ml of absolute methanol in two separate containers and labelled. One of the solutions was used in staining without adding a mordant while on the other hand, the second solution was mixed

with ferric chloride as a mordant and used for staining, both at varying time (10, 20, 30mins). Both solutions were also used separately and interchangeably as primary and counter stains in conventional Gram staining technique. Stained bacterial smears were rinsed and allowed to dry and viewed using x100 objective lens with immersion oil for the characteristics and differentiation of varying bacterial cells.

Experimental Procedures

Bacterial smears (*Staphylococcus aureus, Escherichia coli* and *Klebsiella pneumoniae*) were made on clean grease free slides and heat fixed. Smeared slides were passed through the aforementioned staining solutions and Gram staining technique while replacing the primary stain (crystal violet) and the secondary stain (neutral red) interchangeably. Smeared slides were exposed to the extract solutions at varying time (10, 20 and 30mins). Control slides were also prepared and stained by Grams staining method. A total of 162 slides were stained with the six solutions (HAAE solution A & B, UAAEI solution A & B, and UAAEII solution A & B). Fifty-four (54) slides each were used to demonstrate *E. coli, S. aureus* and *K. pneumoniae* smears sub-divided into 18 slides from each organism. Three (3) slides each were stained with the original solution A and B at varying time (10, 20 and 30mins). Six (6) slides each were stained interchangeably as primary and counter stains representing as the modified Gram staining technique. Control slides were also prepared and stained by Gram's Staining Method. The hydrogen ion concentration (pH) of the varying solutions prepared from aloe vera leaf extract was determined using pH paper. Photomicrographs were taken using a light microscope with a digital camera attached to it, giving a micrographic representation of the various bacteria cells stained by this *A. barbadensis* dyes.

RESULTS

A brownish-yellow colour which turned black on addition of ferric chloride for oxidation and brown to brownish black on adding ferric chloride as a mordant were observed. Unheated Alcoholic Aloe Extract1 method gave the highest yield of total anthraquinone (Table 1a). The results of the qualitative phytochemical analysis of methanolic leaf extract of *A. barbadensis* indicated the presence of the active constituent anthraquinone. The hydrogen ion concentration of the different solution prepared in this study was acidic; 5.0 and 3.0 (Table 1b). Staining characteristics of the various extracts on bacterial cells suggested that the *A. barbadensis dye* had affinity for both Gram positive and Gram negative bacteria although there was no differentiation (Table 2-4). Meanwhile, the staining efficiency of all the solutions prepared from the different extracts in this study is represented in Plate 1-3.

Table 1a: Content of Anthraquinone in the *Aloe barbadensis* Extracts

Extraction Method	Anthraquinone content (g)
Heated Alcoholic Aloe Extract (Methanol/Chloroform)	10.0
Unheated Alcoholic Aloe Extract I (Methanol/Chloroform)	4.84
Unheated Alcoholic Aloe Extract II (Methanol)	18.8

1b: Hydrogen ion concentration of the varying solutions of *A. barbadensis* leaf extract

Extract	pH-value
Heated alcoholic Aloe Extract in absolute alcohol	5.0
Heated alcoholic aloe extract solution with ferric chloride	3.0
Unheated Alcoholic Aloe Extract I in absolute alcohol	5.0
Unheated Alcoholic Aloe Extract I solution with ferric chloride	3.0
Unheated Alcoholic Aloe Extract II in absolute alcohol	5.0
Unheated Alcoholic Aloe Extract II solution with ferric chloride	3.0

Table 2: Staining Characteristics of Heated Alcoholic Aloe Extract on Bacterial Cells

Staining characteristics	HAAE Without ferric chloride	HAAE with ferric chloride	HAAE as primary stain	HAAE as counter stain	HAAE with ferric chloride as primary stain	HAAE with ferric chloride as counter stain
Component affinity						
E.coli	GNB	GNB	GNB	GNB	GNB	GNB
K.pneumoniae	GNB	GNB	GNB	GNB	GNB	GNB
S. aureus	GPC	GPC	GPC	GPC	GPC	GPC
Staining efficiency						
E.coli	++	++	++	++	++	++
K.pneumoniae						
S. aureus						
Differentiation						
E.coli						
K.pneumoniae	ND	ND	ND	ND	ND	ND
S. aureus						

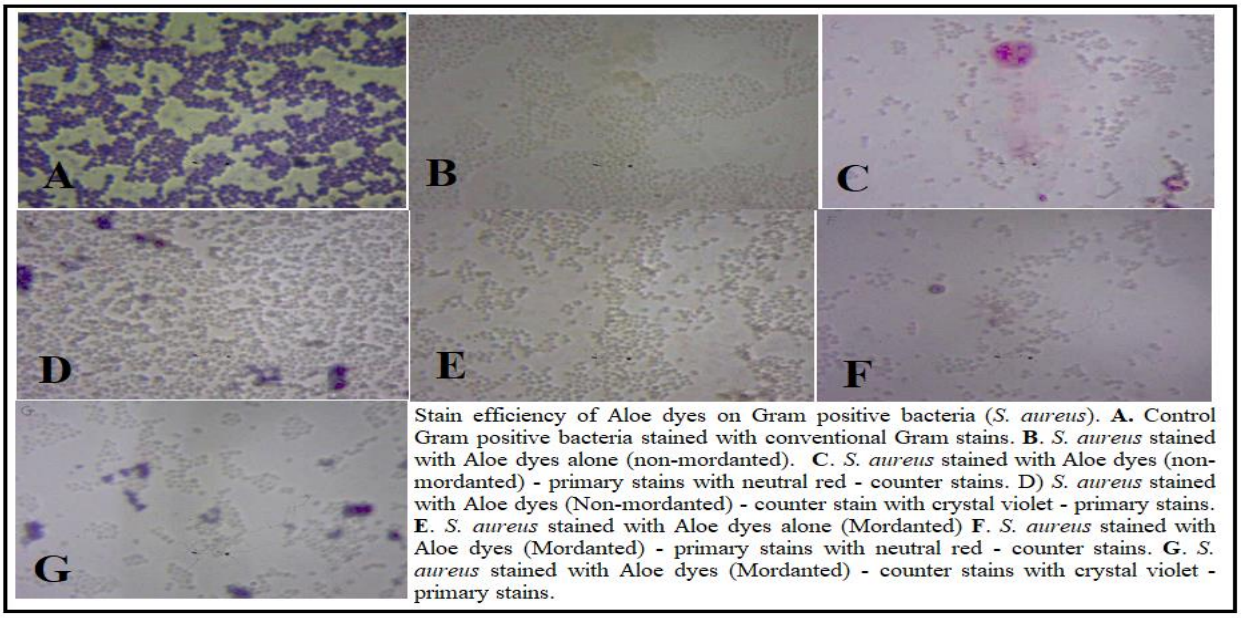
Table 3: Staining Characteristics of Unheated Alcoholic Aloe Extract1 on Bacterial Cells

Staining characteristics	UAAE1 Without ferric chloride	UAAE1 with ferric chloride	UAAE1 as primary stain	UAAE1 as counter stain	UAAE1 with ferric chloride as primary stain	UAAE1 with ferric chloride as counter stain
Component affinity						
E.coli	GNB	GNB	GNB	GNB	GNB	GNB
K.pneumoniae	GNB	GNB	GNB	GNB	GNB	GNB
S. aureus	GPC	GPC	GPC	GPC	GPC	GPC
Staining efficiency						
E.coli	++	++	++	++	++	++
K.pneumoniae						
S. aureus						
Differentiation *E.coli*						
K.pneumoniae						
S. aureus	ND	ND	ND	ND	ND	ND

Table 4: Staining Characteristics of Unheated Alcoholic Aloe Extract11 on Bacteria Cells

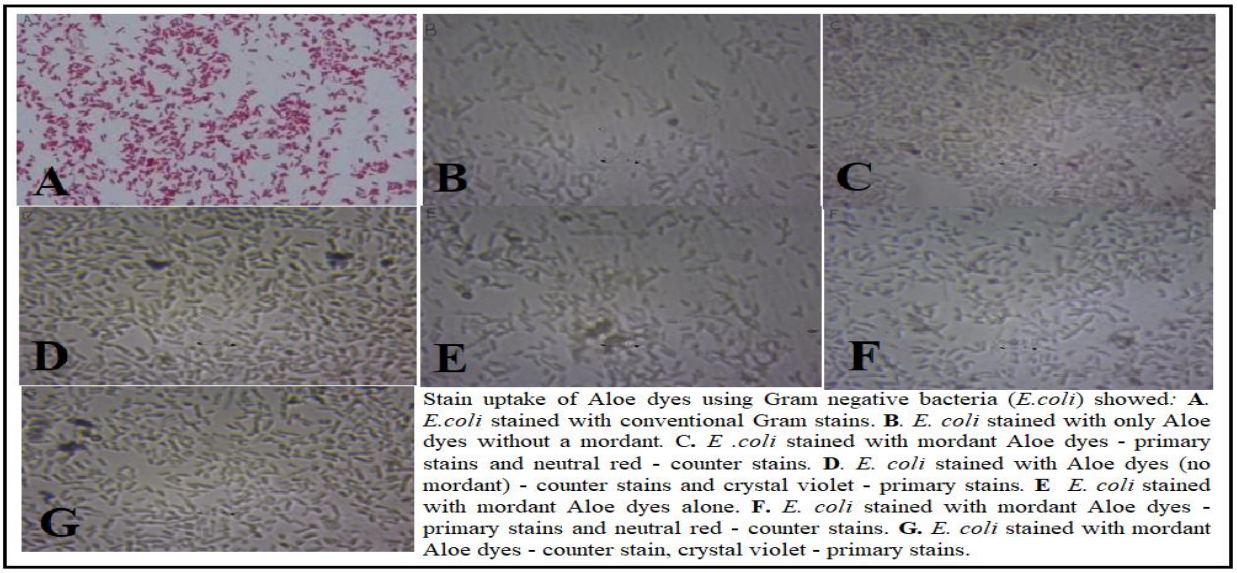
Staining characteristics	UAAEII Without ferric chloride	UAAEII with ferric chloride	UAAEII as primary stain in Gram stain	UAAEII as counter stain in Gram stain	UAAEII with ferric chloride as primary stain	UAAEII with ferric chloride as counter stain
Component affinity						
E.coli	GNB	GNB	GNB	GNB	GNB	GNB
K.pneumoniae	GNB	GNB	GNB	GNB	GNB	GNB
S.aureus	GPC	GPC	GPC	GPC	GPC	GPC
Staining efficiency						
E.coli	++	++	++	++	++	++
K.pneumoniae						
S.aureus						
Differentiation						
E.coli	ND	ND	ND	ND	ND	ND
K.pneumoniae						
S.aureus						

Keys: ND = No Differentiation, GPC = Gram Positive Cocci, GNB = Gram Negative Bacilli; HAAE = Heated Alcoholic Aloe Extract; UAAEI = Unheated Alcoholic Aloe Extract I; UAAEII = Unheated Alcoholic Aloe Extract II; ++ Efficient.



Stain efficiency of Aloe dyes on Gram positive bacteria (*S. aureus*). **A.** Control Gram positive bacteria stained with conventional Gram stains. **B**. *S. aureus* stained with Aloe dyes alone (non-mordanted). **C**. *S. aureus* stained with Aloe dyes (non-mordanted) - primary stains with neutral red - counter stains. D) *S. aureus* stained with Aloe dyes (Non-mordanted) - counter stain with crystal violet - primary stains. **E**. *S. aureus* stained with Aloe dyes alone (Mordanted) **F**. *S. aureus* stained with Aloe dyes (Mordanted) - primary stains with neutral red - counter stains. **G**. *S. aureus* stained with Aloe dyes (Mordanted) - counter stains with crystal violet - primary stains.

Plate 1: Demonstration of Aloe dyes on *S. aureus* at various staining duration (x100)



Stain uptake of Aloe dyes using Gram negative bacteria (*E.coli*) showed: **A**. *E.coli* stained with conventional Gram stains. **B**. *E. coli* stained with only Aloe dyes without a mordant. **C.** *E .coli* stained with mordant Aloe dyes - primary stains and neutral red - counter stains. **D**. *E. coli* stained with Aloe dyes (no mordant) - counter stains and crystal violet - primary stains. **E** *E. coli* stained with mordant Aloe dyes alone. **F.** *E. coli* stained with mordant Aloe dyes - primary stains and neutral red - counter stains. **G.** *E. coli* stained with mordant Aloe dyes - counter stain, crystal violet - primary stains.

Plate 2: Demonstration of Aloe dyes on *E. coli* at various staining duration (x100)

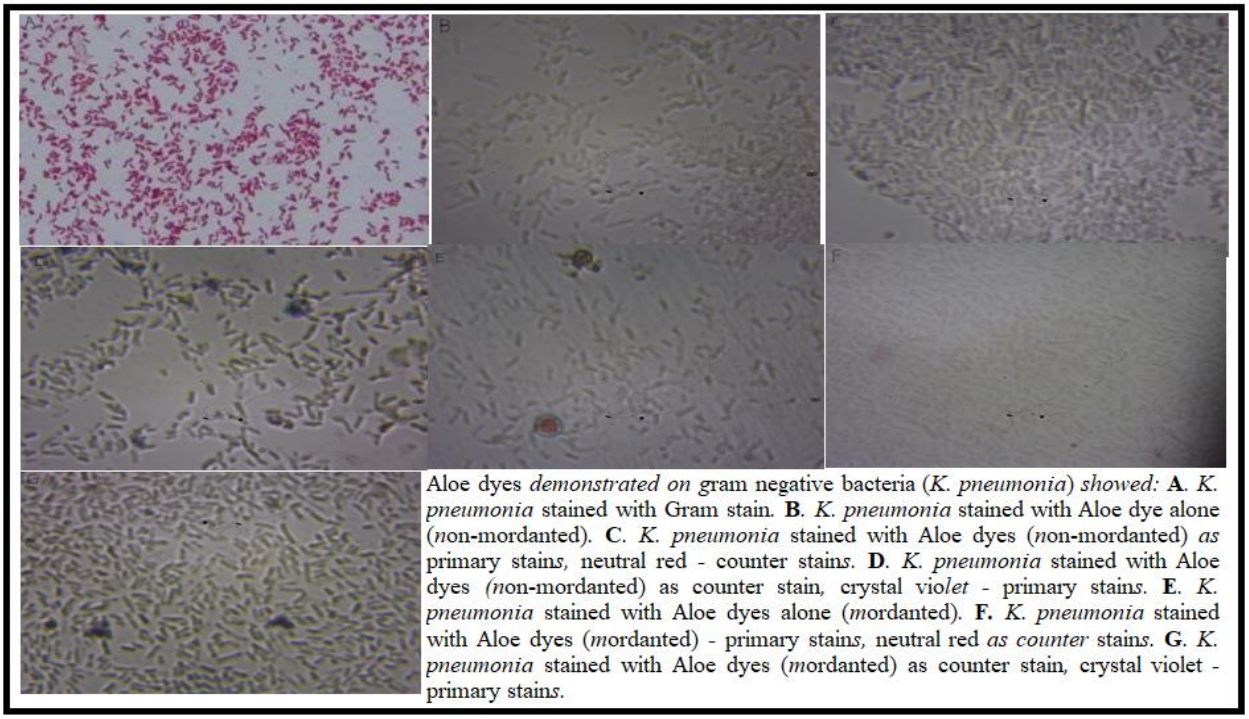


Plate 3: Demonstration of Aloe dyes on *K. pneumoniae* at various staining duration (x100). Note: all slides showed same staining uptake with the different solutions, time and extraction methods for both gram positive or negative bacteria.

DISCUSSION

Gram staining is a technique used in the differentiation of two large groups of bacteria on the basis of their different cell wall constituents. Gram positive bacteria absorb the primary dye (crystal violet) as a result of the thick layer of peptidoglycan in their cell walls, which retains the primary dye used in staining these bacterial cells. On the other hand, Gram negative bacteria absorb the counter stain (neutral red) due to the thinner peptidoglycan cell wall, which does not retain the crystal violet during the decolourizing process (Tripathi and Sapra, 2021). Three processes are involved in Gram staining reaction; water-soluble dye called crystal violet, which is the primary stain, with the addition of iodine (forming a complex between the crystal violet and iodine) and acts as a mordant that fixes crystal violet to bacterial cell wall, decolourization with acetone which dehydrates the peptidoglycan layer, shrinking and tightening it and then counterstaining with safranin, carbol fuschin or neutral red (Ryan and Ray, 2004; Tripathi and Sapra, 2021).

In the staining of bacterial cells using the newly compounded extract as primary and counter stains, Gram positive (cocci) and Gram negative (bacilli) bacterial cells absorbed the brown coloration of aloe dye displacing both crystal violet and neutral red with no differentiation of cells, which is contrary to Gram staining principle that requires gram positive bacteria to retain the color of the primary stain while gram negative retains the color of counterstains (Ryan and Ray, 2004). There is no disruption in the purplish coloration of the Gram positive cells due to the lighter coloration of the counter stain in comparison with the primary stain (crystal violet). The decolorized Gram negative cells are stained red nonetheless. Our results aligned with the aforesaid characteristics by Ryan and Ray (2004) except that there is no structural differentiation of the organism. Though, bacterial cells are visibly stained and identified with the brown aloe dye coloration. Lack of differentiation in this research might be due to differences in ionic charges between conventional Gram stains (crystal violet and

neutral red) which are cationic (basic) compared to *A. barbadensis* dye which is anionic (acidic). Gaurab (2017); reported that bacterial cells are negatively charged due to large number of proteins with COO- group resulting to repulsion of same charges, which accounts to no differentiation in this study as the chromogen of the present dye (anionic stain) is negatively charged.

The pink/red color in the ammonia phase of the test for anthraquinones, which is indicative of its existence conformed to Gritsanapan and Mangmeesri (2009) report on standardized *Senna alata* leaf extract. Solutions prepared from these extracts stained bacterial cellss shades of brown in different time at room temperature. At 10 minutes there was visible colour impartation which had similar staining uptake as those stained at 20 and 30 minutes. The dual role of the present extract as counterstain in place of neutral red and primary stain for crystal violet showed that the gram negative and gram positive bacterial cells both absorbed the brown color of aloe dye displacing the purplish and pinkish colours exerted by crystal violet and neutral red with no visible differentiation plate, which also agreed with Ryan and Ray (2004). The hydrogen ion concentration of the different solutions were acidic; the non-mordanted solution gave a reading of 5.0 which is a close value as the aloe pH (4.5) stated in a study (Shariff and Sandeep, 2011) while the addition of a mordant (ferric chloride) reduced the pH to 3.0. However, our study, showed visible colour impartation of structural appearance of organisms whether coccus or bacillus and according to Tripathi and Sapra (2021), describing bacteria as cocci or spheres distinguish it from bacilli or rods, which are the first of many taxonomic traits for identifying and classifying a bacterium according to binomial nomenclature.

Based on their different interactions with dyes and stain uptake in this study, the difference in the staining time between the conventional stains (crystal violet and neutral red) which was left for 1 minute and the *A. barbadensis* dye which was left for 10, 20 and 30 minutes respectively could be a factor. This prolonged timing could have resulted in over staining, countering the efficacy of the crystal violet and neutral red used in the staining technique employed in this study. Generally, extracts from all extraction methods used in this study showed similar staining efficiency; the differences in the pH of the solutions prepared (mordanted and non-modanted) did not show substantial qualitative staining differences in staining bacterial cells.

CONCLUSION

The dye of *A. barbadensis* has been evaluated for the first time as far as this study can verge. The plant contains a bioactive agent that imparts colour with similar staining properties as the conventional Gram staining technique. The use of this extract as a single dye for simple staining showed its effectiveness as a dye as it visibly imparted colours on cells / organisms. We suggest that an alkaline buffer be added and adjusted according to cellular staining characteristics to further enhance the bacteriologic staining uptake of the present extract.

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Conflicting Interest

No conflict of interest is associated with this manuscript.

Authors Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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