The utilization of methanolic *Bixa orellana* (Annatto) seed extract as substitute for safranin in Gram staining

SAM DANIEL T. VENTURINA, JOSH ZAVION A. COMUELO, WAYNE ROCQ A. SAMANIEGO, and FERNANDO CHRISTIAN JOLITO III

Philippine Science High School - Western Visayas Campus, Brgy. Bito-on, Jaro, Iloilo City 5000, Department of Science and Technology, Philippines

Abstract

The routine use of safranin, a synthetic dye, in Gram staining raises a number of environmental and safety concerns. This study evaluates the utility of the methanolic extract of *Bixa orellana* (Annatto) seeds as replacement for safranin as counterstain in Gram staining. Pure and mixed bacterial smears of *Escherichia coli* and *Staphylococcus aureus* were stained with Annatto and safranin. The staining showed that the *Bixa orellana* extract did not stain the bacteria in any of the slides. Results show that the pH of the extract was found to be acidic at 5.92 in comparison to safranin, which was basic at 7.44. The inability of the extract to stain the bacteria may be attributed to its acidic pH which makes it unable to bind to the acidic cell wall of the bacteria.

Keywords: *annatto, bixin, safranin, Gram staining, methanol*

Introduction. At present, due to increasing environmental consciousness among commercial dyers and small textile exporters, interest has shifted towards the utilization of naturally-sourced colour pigments in food materials, pharmaceuticals and textiles, as an alternative to their synthetic counterparts [1]. Although synthetic dyes are produced from cheap sources and show superior fastness properties, their usage cause pollution and can be harmful to humans [2]. Natural dyes, in contrast, are less hazardous and eco-friendly [3].

Dyes are used in Gram staining, which is a process which uses basic stains to impart color to bacteria [4]. It uses a primary stain and a counterstain to differentiate gram-positive and gram-negative bacteria. However, commonly used stains such as safranin and crystal violet are synthetically made. Gram staining is primarily used in histopathology for primary identification of bacteria, as such information is useful in deciding the appropriate treatment for patients [5].

Unlike most organic compounds, dyes possess color because they absorb light in the visible spectrum (400–700 nm), have at least one chromophore or colour-bearing group, have a conjugated system, i.e. a system of connected p orbitals with delocalized electrons in a molecule, and exhibit resonance of electrons, which is a stabilizing force in organic compounds [6]. Stains are examples of a dye which has the ability to impart colour to tissues. However, the pH of a stain affects its ability to adhere to a specific tissue thus, basic structures have better affinity towards acidic stains while acidic structires have better affinity towards basic stains due to their nature.

B. orellana (Annatto), a dye yielding plant, is known for its lack of toxicity and its high tinctorial value [7]. Annatto dyes are widely used in the food, pharmacological and cosmetic industries due to the intensity of their colours, their greater stability and the wide variety of tones [8]. According to a study by Lauro [9], bixin, the liposoluble component of Annatto, amounts to about 80% of the plant's carotenoids.

Bixin, the main colorant found in the seeds of *Bixa orellana*, is an example of a dye with the capacity to absorb light. Studies have shown that bixin is soluble in organic solvents with medium polarity [10]. The studies conducted by Attokaran [11], Rahmalia et al. [12], and Scotter [13] have explained that bixin is soluble to most polar organic solvents. A study conducted by Braide et al. [14] using methanolic crude extract of *Bixa orellana* with NaOH and glacial acetic acid was unable to stain bacteria. Methanol is a suitable reconstituting agent and solvent for extraction because a study conducted by Husa et al. [15] found that methanol results in the highest bixin yield in comparison with water, hexane, and acetone due to its ability to dissolve the cell membrane in comparison to the other aforementioned solvents.

The aforementioned hazards that synthetic dyes possess [2] provides an avenue for exploring alternatives. While there has been research on using plant-based dyes as alternatives to commercial bacterial stains [14], the utility of bixin from *Bixa orellana* as a replacement for safranin has not been explored.

The present study proposed to investigate *Bixa orellana*, for its potential in being a substitute for safranin in Gram staining. It specifically aimed to:

(i) compare the UV-Spectra of the *Bixa orellana* extract at 50ppm to Bixin;

(ii) compare the pH of the stock solutions of both *Bixa orellana* and safranin; and

(iii) compare the staining capabilities (visibility and color intensity) of both Annatto and safranin.

If the *Bixa orellana* extract is proven to be a dye comparable to safranin in terms of effectiveness as a bacterial stain, it would be beneficial to the

How to cite this article:
CSE: Venturina SD, Comuelo JZ, Samaniego WR, Jolito FC. 2020. The utilization of methanolic *Bixa orellana* seed extract as substitute for safranin in Gram staining. Publiscience. 3(1):33-36.
APA: Venturina, S.D., Comuelo, J.Z., Samaniego, W.R., & Jolito, F.C. (2020). The utilization of methanolic *Bixa orellana* seed extract as substitute for safranin in Gram staining. *Publiscience,* 3(1):33-36.

environment, as the use of plant dyes as an alternative can lead to more environmentally-friendly practices.

Methods. The study was conducted to evaluate the potential of the *Bixa orellana* methanolic extracts as a substitute to safranin. Bixin, one of the primary dye components of *Bixa orellana* extracts, was extracted utilizing methanol (CH_3OH) as the solvent. For the preparation of *Bixa orellana*, the seeds were collected then ground. The seeds were then macerated in methanol and stirred using a magnetic stirrer. The extracts were then placed in a rotary evaporator to remove the methanol solvent and to acquire crude extract. Gram staining was then performed using *S.aureus* and *E. coli* as test organisms. The stains were viewed using a Digital Microscope and evaluated their staining capability in comparison to safranin.

Preparation of Materials. The *Bixa orellana* seeds were separated by hand then washed with distilled water and sterilized in a hot air oven at 60˚C for 24 hours. The seeds were then crushed using a blender.

Extraction. One hundred grams (100g) of the pulverized seeds were added to 1 L of methanol (CH_3OH) and stirred for 12 hours using a magnetic stirrer and stored without sunlight at room temperature for another 12 hours. The extract was then filtered using Whatman Filter Paper No. 1 and was placed in an IKA RV10 Rotary Evaporator at 40 ˚C until the methanol had leaving solid crude extract remained. The extract was then stored in a refrigerator prior to staining.

Test for pH. Crude extract of mass 0.25g was dissolved in 10 mL of methanol (CH_3OH). Its pH was then measured using a pH meter.

UV-Vis Spectroscopy. Crude extract of mass 0.25g was dissolved in 10 mL of methanol (CH_3OH). The absorbance of the reconstituted solutions was then measured with a UV-Vis spectrophotometer at 300-650 nm and compared to the expected absorption maxima from the study of Silva et al. [16].

Reconstitution of extract. Crude extract of mass 2.5g was dissolved in 50ml of methanol to form the solution.

Staining. A drop of normal saline solution was placed on the slide. Using an inoculating needle, the cultured bacteria was smeared on the slide and allowed to dry. The slide was then passed quickly over the flame of an alcohol lamp three times. The slide with the heat fixed smear was then flooded with crystal violet for one minute and was then rinsed with distilled water. The smear was gently flooded with Gram's iodine for one minute and was then rinsed with distilled water. The smear was then decolorized using a 50 v/v% mixture of acetone and alcohol. The slide was then flooded with safranin as counterstain and left to stand for one minute. The slide was then rinsed with distilled water [17]. The process was repeated using the methanol-reconstituted *Bixa orellana* extract as a counterstain. For safranin, 30 slides consisting of 10 slides of mixed *E. coli* and *S. aureus* bacteria, 10 slides of *S. aureus*, and 10 slides of *E. coli* were stained. Another 30 of slides were stained with *Bixa orellana* extract as counterstain instead of safranin.

Safety procedure. The use of personal protective equipment such as laboratory gowns, safety goggles, gloves and masks in the laboratory was observed at all times. The solvents and bacteria were stored in sealed containers with proper labels for clear identification. The materials used to handle *S. aureus* and *E. coli* were sterilized in an autoclave prior to use. The handling of bacteria was performed in a biosafety level 2 laboratory. The bacteria cell cultures were properly labeled and stored in an incubator.

Results and Discussion. The study aimed to investigate the use of *Bixa orellana* methanolic extract as a substitute for safranin in Gram Staining. Specifically, it aims to compare the pH of the stock solutions of both *Bixa orellana* and safranin, compare the UV-Spectra of the bixin extracts, and compare the staining capabilities of both *Bixa orellana* and safranin. 60 slides were stained and evaluated. The two stains: *Bixa orellana* extract and Safranin were used to stain the bacteria. The slides were evaluated and compared.

Measurement of pH. The pH of the Methanol extract was found to be acidic at 5.92 while the safranin solution was found to be basic at pH 7.44. The ability to stain certain structures is determined by the pH values of the stain. Safranin is an example of a basic dye, which easily release OH^- ions and readily accept H^+ ions, this leaves the stain positively charged and ready to adhere to negatively charged molecules like the polyphosphated nucleic acids and proteins found in the cell wall of bacteria [18]. The results suggest that the *Bixa orellana* extract was not able to stain the bacteria because of its acidic pH, which is in agreement with Prescott et al. [17], which posits that the pH of the dye may alter staining effectiveness since the nature and degree of the charge on cell component changes with pH. It then follows that acidic structures are stained by basic dyes while basic structures are stained by acidic dyes [19]. This is also in agreement with a study conducted by Chukwu et al. [20], wherein modified Henna extracts with acidic pH were unable to stain the bacterial cells while Henna extracts oxidized with potassium permanganate had a better counter staining reaction due to its neutral pH as stated in the study.

UV-Vis Spectroscopy. In Fig. 1, the UV–Vis absorbance spectrum is shown for the extract reconstituted with methanol. The absorption peaked at 425.64nm, which is in good agreement with the expected absorption maxima for bixin which are at 429 nm, 457 nm, and 487 nm [14]. This indicates that bixin was extracted. The presence of bixin indicates that the methanolic extract has the potential to stain bacterial cells because unlike most organic compounds, bixin, is an example of a dye because it has a chromophore, which is characterized by a conjugated system and extended delocalized system [21]. Bixin also contains an auxochrome, which are groups that increase the color absorption of a chemical, represented by the hydroxyl group [21].

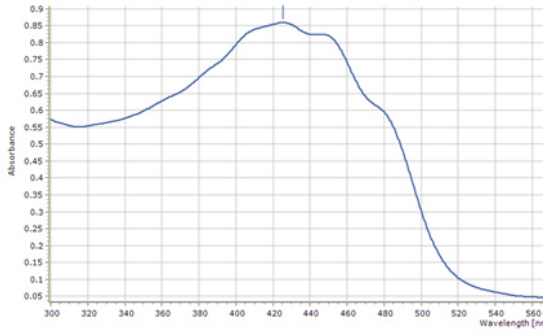
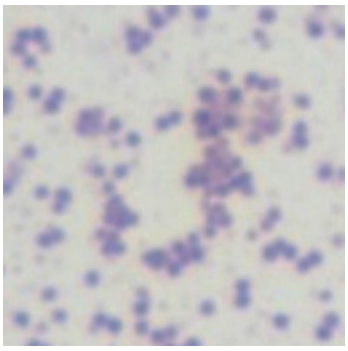
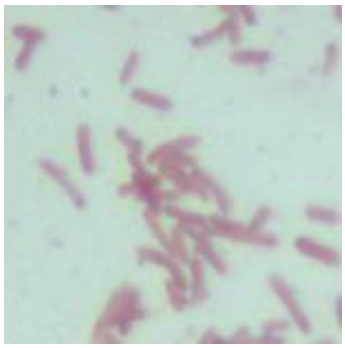
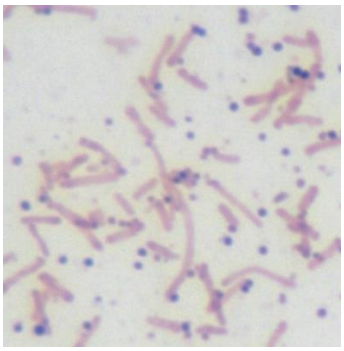
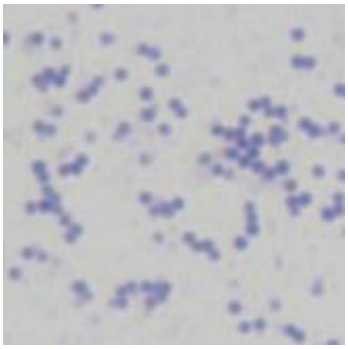
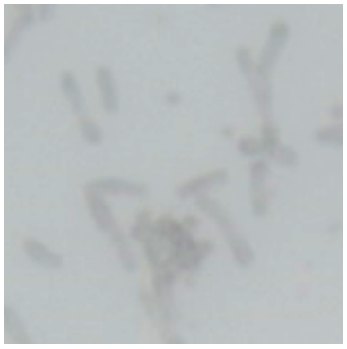
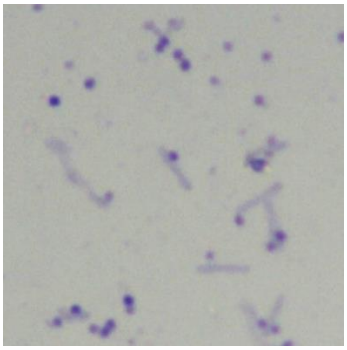


Figure 1. The graph of the UV-Vis Spectra for the methanolic *Bixa orellana* extract.

Staining. The evaluation of mixed bacterial smears as shown in Table 1 showed that *S.aureus* counterstained with Annatto stain had no traces of color other than the violet from the crystal violet stain. This conforms with the expected coloration for the Gram Staining of *S.aureus.* Gram-positive bacteria are expected to retain the color of the primary stain with no traces of the counterstain because it has a thicker peptidoglycan layer which shrinks and traps the primary stain-mordant complex during decolorization [20]. Gram-positives possess cell membranes with low permeability to iodine in alcoholic solution. This, combined with the low alcohol solubility of the dye-iodine compound, can be the cause of the retention of the primary stain [4]. Gram-negative bacteria is expected to be colorized by the counterstain as its thinner peptidoglycan layer is unable to retain the primary stain-mordant complex during decolorization while the counterstain binds itself to the bacteria [4]; however, the *E.coli* in the mixed bacterial smears counterstained with the acidic Annatto stain appeared to be decolorized showing no color compared to the sample counterstained with safranin which was stained red due to its inability to bind to the bacterial cell wall.

Table 1. *S.aureus* and *E.coli* samples stained using safranin and the methanolic *Bixa orellana* extract.

	S. aureus	*E.coli*	Mixed
Safranin			
Bixa orellana extract			

Limitations. The study is limited to the assessment of the staining capability of *Bixa orellana* extract with the bacteria *E. coli* and *S. aureus,* and its comparison to safranin utilizing pH, UV-Vis color spectra, and staining capability as parameters for the comparison. No other test organisms were used in this study. Only the methanolic extract was used to stain the test organisms. For staining, only one licensed medical technologist evaluated the slides. The actual size of the bacteria was not measured.

Conclusion. The methanolic extract of *Bixa orellana* (Annatto) was investigated for its use as an eco-friendly alternative to safranin in Gram staining. The Annatto extract failed to stain the *E. coli* samples, which remained colourless after staining. The acidic pH of the Annatto stain had no staining affinity with the acidic structure of the bacterial cell wall. Conversely, safranin, a basic stain, had better staining affinity towards the acidic structures of *E.coli*. The *Bixa orellana* extracts therefore cannot replace safranin as a counterstain for Gram staining. However, due to its capacity to absorb light as a dye, it has the potential to be used as a stain. This can be explored by future studies given that it is adjusted to have the optimal parameters of a bacterial stain.

Recommendations. The replication of this study with more focus on the pH of the stain is recommended, so that the relationship between the pH and the staining capability of the extract can be better observed. The pH of the Annatto stain was not adjusted as the study focuses on baseline parameters. This may have been a factor to the results of the staining. It is therefore possible that the adjustment of the pH, through addition of treatments, may yield positive results. For future research, we recommend the use of other plant extracts for Gram staining. More concentrations may be used in order to further test their capabilities as an alternative stain.

Acknowledgement. The authors would like to acknowledge the University of San Agustin MedTech Lab for providing the necessary materials for the study.

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