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The use of *Clitoria ternatea* (blue ternate) ethanolic extract as a potential stain for bacteria

CHERIL B. TRIOL, ATHENA THERESE V. DIONELA, ANGELA ISABELLE D. ECUBE, and CATHERINE JOY A. MEDIODIA

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

Abstract

Most synthetic microbial stains are pollutants that pose carcinogenic, mutagenic and teratogenic health risks. Thus, this study aimed to investigate the ability of *Clitoria ternatea* ethanolic extract as a potential stain for bacteria. The extract was utilized in the simple staining and Gram staining of *Staphylococcus aureus*, *Escherichia coli*, and mixture of the two. The properties of the stain such as presence of anthocyanin, pH level, color, and solubility of the extract were determined. The staining ability of the extract, in terms of visibility of cell walls and color intensity, was determined through frequency count that showed that majority of the replicates had defined cell walls with incomplete uptake of stain in simple staining and Gram staining. *Clitoria ternatea* ethanolic extract has the potential to impart stain on *Staphylococcus aureus* and *Escherichia coli* bacteria through simple staining and Gram staining; however, due to the incomplete uptake of stain by the bacteria, it cannot be considered as an alternative to commonly-used bacterial stains. Hence, the researchers recommend the development of a new staining technique for the potential stain.

Keywords: *Clitoria ternatea, dyes, stain, anthocyanin, ethanolic extract*

Introduction. Dyes, natural or synthetic, are substances that are soluble in a medium and are usually used to give a desired color to non-food materials [1,2,3,4], such as animal and plant tissues, and microbes to make them visible and distinct [5,6]. Microorganisms viewed under the microscope need to be fixed and stained to improve visibility, emphasize morphological features, and sometimes preserve them [1]. In microbiology, this process is known as staining [3].

In staining bacteria, bacterial smears are initially made to fix bacteria on the slide [1]. Most stains for bacteria are cationic as they bind to negatively-charged structures such as the bacterial cell wall. Because of the opposite charges of both the stain and bacterial cell wall, the stain adheres to the surface of the bacterial cells [7]. Currently, most microbial stains in use are chemically synthesized due to its convenience [4,8]. However, they pose a threat to the environment and human health [9] as some synthetic dyes contain allergenic components [3,4] and toxic heavy metals, contributing to land, water and air pollution [3]. For example, crystal violet, a dye that has been extensively used as a biological stain, is regarded as a toxic biohazard substance that causes serious environmental and health problems [2].

Because of this, researchers are searching for alternative dyes for staining microbial cells, which are not hazardous to living things [3,9]. Research show that extracts for the production of dyes can be obtained from natural sources such as plants [3,6,10], animals, and the soil [4]. According to recent studies, natural dyes from plants are used as histological stains for tissue components [3,9]. These natural dyes are known to be convenient, cheaper, safe, non-toxic, eco-friendly, renewable and biodegradable [3,6].

Plant extracts contain natural phenolic compounds that are structurally related to a family of water-soluble pigments known as anthocyanins [11]. Anthocyanin compounds are flavonoids found in the flower petals, fruits and leaves of several plants, and are known to be the plant's main colorant molecule [12]. It contains flavylium cation that is its chromophore [13]. The color stability of these compounds depends on several factors such as chemical structure and pH [13,14]. Cationic or basic stains are called so because their coloring agent is located in the basic part of the compound while the acidic radical is inactive [15]. They also carry a positive charge and stains negatively-charged elements [16]; thus, many of them are considered as Lewis acids or electron acceptors [17]. On the other hand, anionic or acidic stains have their coloring agent located in the acidic part of the compound [15], and they carry a negative charge that stains positively-charged elements [16]. Thus, many of them are considered as Lewis bases or electron donors [17]. With anthocyanins having a positive charge [11], they are considered to be the key compound causing the staining ability of several plant extracts [18], especially in staining negatively-charged structures such as those of bacterial cell walls. Thus, plants with the presence of anthocyanin can potentially be a source of natural histological stains.

Clitoria ternatea, commonly known as blue ternate, is a strangling and climbing herb commonly used as a medicinal plant due to its wide range of pharmacological activities and phytochemicals, including anthocyanin [11,18]. Anthocyanin

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compounds of blue ternate plants are commonly found in their flowers as they are responsible for the flowers' red violet-blue color [14]. The ability of *Clitoria ternatea* as a stain is not much given focus and studies on it are limited. A study conducted by Suebkhampet and Sotthibandhu [11] utilized the aqueous crude extract of *Clitoria ternatea* as a stain for blood smears; however, its capacity to stain microorganisms such as bacteria is yet to be explored.

This study aimed to investigate the ability of the ethanolic extract of *Clitoria ternatea* flowers as a potential stain for bacteria. Specifically, it aimed to:

(i) test the ethanolic extract of *Clitoria ternatea* for the presence of anthocyanin;

(ii) determine the properties of ethanolic extract of *Clitoria ternatea* such as (a) pH level, (b) color, and (c) solubility, and compare them to the properties of crystal violet, a conventional bacterial stain;

(iii) use the ethanolic extract of *Clitoria ternatea* to stain Gram-positive *Staphylococcus aureus*, Gram-negative *Escherichia coli*, and mixture of the two bacteria using the simple staining and Gram staining method; and

(iv) evaluate the ability of ethanolic extract of *Clitoria ternatea* to stain Gram-positive *Staphylococcus aureus*, Gram-negative *Escherichia coli*, and mixture of the two bacteria in terms of (a) visibility of cell walls, and (b) color intensity.

Methods. This is an exploratory study. *Clitoria ternatea* flowers were oven-dried, macerated in ethanol and filtered. The crude extract was obtained and reconstituted with ethanol. The properties of the extract were determined and compared to crystal violet. The ethanolic extract and crystal violet was used to stain S*taphylococcus aureus, Escherichia coli*, and mixture of the two bacteria through simple and Gram staining. Positive control using crystal violet and negative control without any stain were also prepared. The staining ability of both the ethanolic extract and crystal violet were evaluated by a licensed professional using a modified rubric.

Ethanolic Extraction. The collected *C. ternatea* flowers were separated petal by petal, washed with distilled water and oven-dried at 75°C for 24 hours [12]. The powdered petals were macerated in 95% ethanol with 1:10 mass to volume ratio. The mixture was filtered using No. 41 Whatman filter paper and subsequently filtered using No. 1 Whatman filter paper [11]. The filtered extract was then subjected to rotary evaporation to obtain the crude extract, which was dissolved again in 95% ethanol in a 1:1 mass-of-extract-to-volume-of-solvent dilution ratio.

Test for the Presence of Anthocyanin. The ethanolic extract of *C. ternatea* was tested for the presence of anthocyanin. A change from the original color of the extract to orange-red to blue-red color upon addition of 1% (v/v) hydrochloric acid (HCl) was used as an indicator for the presence of anthocyanin in the extract [19].

Assessment of Physical and Chemical Properties. The following properties were assessed: pH, color, and solubility. The pH values of the ethanolic extract of *C. ternatea* and crystal violet were measured using a pH meter. The color of both stains were determined based on the Pantone Colour Matching System. For the solubility of ethanolic extract in water, one mL of distilled water was added to one mL of ethanolic extract, and the solution was checked if both liquids are miscible where there is no distinction between the two liquids in the mixture.

Preparation of Bacterial Smears and Staining. *Staphylococcus aureus, Escherichia coli*, and mixture of the two bacterial smears were prepared in a biosafety cabinet. The ethanolic extract was used as primary stain for the experimental set-up in the simple staining of *S. aureus* and *E. coli,* and in the Gram staining of *S. aureus, E. coli* and mixture of the two bacteria [20]. Crystal violet, on the other hand, was used for the positive control. Other parts of the simple staining and Gram staining method were not modified. Negative control without any stain was also prepared.

Data Analysis. The evaluation of the visibility of cell walls of bacteria and their color intensity utilized nominal scale data, with values from zero to three, and are based on a rubric modified from Sridhara et al. [21]. Frequency count was used to describe the stain performance of the extract and of crystal violet in terms of visibility of bacterial cell walls and the color intensity. The bacterial smears were evaluated, and the results were verified by a licensed medical technologist.

Safety Procedure. During the conduct of the research, the use of appropriate personal protective equipment (PPE) was observed, and hand hygiene was performed regularly. Chemicals and bacteria were handled properly according to their MSDS. For the disposal of bacterial smears, all slides were collected, autoclaved and turned over to the science research assistant (SRA) in charge of the laboratory.

Results and Discussion. The ethanolic extract obtained a positive result in the test for the presence of anthocyanin. Both the ethanolic extract and crystal violet have low pH, have different shades of violet in color, and are soluble in water. For the staining ability of ethanolic extract through simple staining, majority of the Gram-positive *S. aureus* and Gram-negative *E. coli* replicates had defined cell walls but with incomplete uptake of stain. For the Gram staining, *S. aureus* and mixture of the two bacterial smears had replicates with defined cell walls but incomplete uptake of stain. *E. coli* replicates had defined cell walls, and the bacteria were properly differentiated.

Presence of Anthocyanin. The color of the ethanolic extract changed from deep violet to deep red, which indicates the presence of anthocyanin in the extract. The use of ethanol as the solvent during extraction optimizes anthocyanin extraction [18], and is reported to perform better compared to using water as the solvent [6,9,21]. Flavylium cation is the basic chromophore of anthocyanin [13]. It is electron deficient and highly reactive [22]. The positive charge in its structure gives it the capacity to bind to

the negatively-charged bacterial cell walls [11,18], thus imparting color to the bacteria. However, several factors may influence the stability of this compound including light, temperature, and the compound's chemical structure and pH [14]. In this study, all factors except pH were controlled.

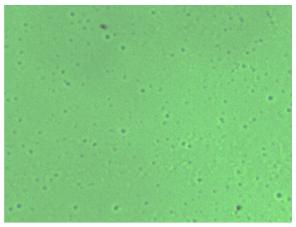


Plate 1. *S. aureus* stained with the ethanolic extract using simple staining.

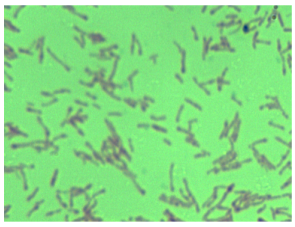


Plate 2. *E. coli* stained with the ethanolic extract using simple staining.

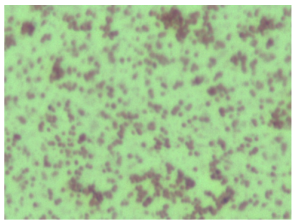


Plate 3. *S. aureus* stained with the ethanolic extract using Gram staining.

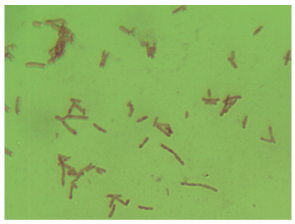


Plate 4. *E. coli* stained with the ethanolic extract using Gram staining.

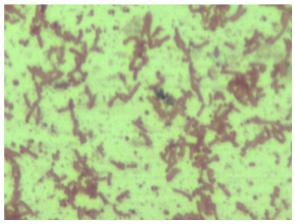


Plate 5. Mixture of *S. aureus* and *E. coli* stained with the ethanolic extract using Gram staining.

pH. Crystal violet staining solution was reported to have a low pH with a value of 5.43 whereas the ethanolic extract of *C. ternatea* was also reported to have a low pH with a value of 4.75.

In staining, the pH of the stain affects the nature and degree of the charge on specific tissue structures [1,4], influencing the ability of the stain to adhere to it [21]. It is known that tissue elements are attracted to the oppositely-charged ions of the stain [4]. The magnitude of the electrostatic charges to be imparted by the dye to the cell component is affected by the pH of the stain [16]. Bacteria are generally stained better with cationic stains due to their anionic cell walls.

Crystal violet is a cationic dye [2,16]. Its major structural form is a monovalent cation that serves as its principal form in solid state and in aqueous solution across a wide range of pH values. As the pH changes, the positive charge in the central carbon atom is delocalized by resonance of the three nitrogen atoms present in the molecule, making it more stable [2]. Crystal violet dissolved in water has a pH range of 2.5 to 3.5 at 10 g/L at 20°C [23]. The reported pH value of crystal violet, which is 5.43, coincides with the expected acidic pH.

The pH value of the ethanolic extract, which is 4.75, is low and acidic. Ethanol, the solvent of extraction, is known to have a slightly basic pH value; however, in the study of Sridhara et al. [21] and Itodo et al. [24], ethanolic extracts are reported to have acidic pH values. This coincides with the acidic pH of the ethanolic extract of *C. ternatea*. A factor that affects the pH of anthocyanin is the structure of the compound, which also influences its color. The flavylium cation in the compound has conjugated double bond that causes the delocalization of the positive charge leading to multiple resonance structures. Anthocyanin at pH 1.0 is red. When pH is between 1.0 and 4.0, discoloration from red to violet occurs, and colorless carbinol base would be formed. This will then undergo water catalyzed-tautomerization to produce chalcone. At pH 4.0 to 6.0, the structure changes into anhydrobase that gives an extension of conjugation to its structure, causing a color change from violet to blue with stronger intensity [14].

Having acidic pH values, both crystal violet and the ethanolic extract of *C. ternatea* are considered as Lewis acids, which make them cationic stains with good staining affinity towards the anionic bacterial cell walls [16,17]. The staining affinity of the acidic ethanolic extract is supported by the study of Sridhara et al. [21] wherein the ethanolic extracts of *Hibiscus* at an acidic pH of 5.7 gave optimal staining in the negatively-charged cytoplasm of tissues. It is also substantiated by the study of Itodo et al. [24] that concludes that the cytoplasmic staining ability of the onion skin extract solution was due to its highly acidic pH.

Color. Crystal violet is known to be blue-violet in color [2]. It is an example of a quinonoid dye with a quinonoid ring as its chromophore and an auxochrome that is responsible for its color and staining properties [15]. It was observed that the corresponding color of crystal violet based on Pantone Colour Matching System is Pantone 2685. The ethanolic extract of *C. ternatea*, on the other hand, was observed to have a deep violet (Pantone 276) color that is from the anthocyanin compound. Both crystal violet and ethanolic extract are violet in color, which is useful for the evaluation of the color intensity of the ethanolic extract as it is relative to the positive control, crystal violet.

The color of crystal violet depends on its acidity. It is yellow at a strongly acidic pH of 0 and is green at a pH of 1.0. When dissolved in water, its color is blue-violet or vibrant purple. The vibrant purple color of crystal violet is caused by the delocalization of the positive charge present in the central carbon atom of crystal violet across the double bonds in the benzene rings that stabilizes the carbonation [2].

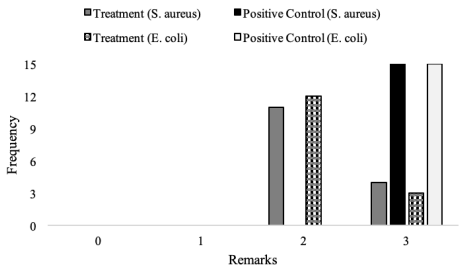
The color of the ethanolic extract also depends on the acidity of anthocyanin present in it [12,14]. Anthocyanin in acidic conditions is red in color [11,14], which explains the change of color of the ethanolic extract from deep violet to deep red upon the addition of the strong acid, HCl. As the pH increases, the structure of the compound changes, and discoloration occurs [14]. The pH value of the ethanolic extract that is 4.75 lies near pH 4.0, and the extract has a violet color. This agrees with the study

of Saptarini et al. [14] that states that at pH 4.0, the color of the anthocyanin compound changes from red to violet. Similar with crystal violet, the color change of the dye is caused by the delocalization of the positive charge present in the molecule across the conjugated double bond that gave some resonance to its structure, thus stabilizing the flavylium cation [14].

The color of both crystal violet and ethanolic extract is influenced by the pH of their dye molecule, and their colors are different at a distinct pH [2,14]. This may be attributed to the difference in their nature and structure. Both stains contain different molecules; thus, they have different spacing of energy levels. This affects the absorption of the visible light radiation in the electromagnetic spectrum that causes dyes to appear colored. This spacing is influenced by the degree of delocalization of the bonding electrons of the molecule [25]. This may cause the different shades of violet in both stains.

Solubility. *C. ternatea* ethanolic extract was soluble in water. It was comparable to crystal violet, which is known to be water-soluble. Due to the positive charge in the flavylium cation of anthocyanin [13], its structure is considered as polar. Thus, the ethanolic extract is soluble in polar solvents such as water [14].

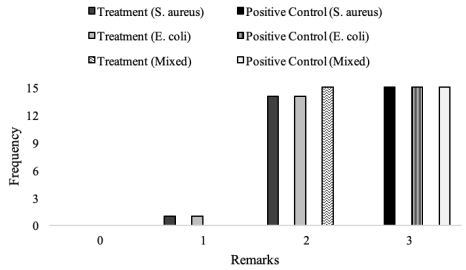
Visibility of Cell Walls. The staining ability in terms of cell wall visibility is based on the uptake of the stain by the bacteria. A complete uptake of the stain means the bacterium shows its regular and expected shape where Gram-positive *S. aureus* must be round and purple whereas Gram-negative *E. coli* must be rod and pinkish-red. For the positive control for simple staining, all replicates of *S. aureus* and *E.coli* had bacterial cells with defined cell walls and complete uptake of stain, as in Figure 1. *S. aureus* bacteria stained with ethanolic extract through simple staining showed that 11 out of 15 replicates had defined cell walls with incomplete uptake of stain whereas only four out of 15 replicates had defined cell walls with complete uptake of stain. The *E. coli* bacteria stained using ethanolic extract through simple staining had 12 out of 15 replicates with defined cell walls but incomplete uptake of stain whereas three out of 15 replicates had defined cell walls with complete uptake of stain.



0 - Cell walls defined and has no color
1 - Cell walls defined but has no color
2 - Cell walls defined with incomplete uptake of stain
3 - Cell walls defined with complete uptake of stain

Figure 1. Staining ability of *Clitoria ternatea* ethanolic extract on *S. aureus* and *E. coli* through simple staining.

For the Gram staining, crystal violet was able to stain all of the replicates for the *S. aureus* and mixture of the two bacterial smears with defined cell walls and complete uptake of stain whereas no trace of crystal violet is visible on all replicates of *E. coli* bacterial smears, as in Figure 2. *S. aureus* stained with ethanolic extract had 14 out of 15 replicates with defined cell walls and incomplete uptake of stain whereas only one replicate had defined cell walls but only exhibits pinkish-red color that can be attributed to the counterstain, safranin. For the Gram staining of *E. coli*, all of the replicates had defined cell walls with proper differentiation between Gram-positive bacteria and Gram-negative bacteria because it exhibited the expected pinkish-red color. All 15 replicates of mixture of the two bacterial smears stained with ethanolic extract through Gram staining had defined cell walls with incomplete uptake of stain.



0 - Cell walls not defined
1 - Cell walls defined and the cells are pinkish-red (*E. coli*) and purple (*S. aureus*)
2 - Cell walls defined with incomplete uptake of stain (both *S. aureus* and *E. coli*)
3 - Cell walls defined and properly differentiated where *S. aureus* cells are purple and *E. coli* cells are pinkish-red

Figure 2. Staining ability of *Clitoria ternatea* ethanolic extract on *S. aureus, E. coli,* and mixture of the two bacteria through Gram staining.

The results showed that the ethanolic extract of *C. ternatea* is a potential bacterial stain because it was able to impart color to the bacteria, although the uptake is incomplete. Its staining ability may be attributed to comparable results between the ethanolic extract and crystal violet in terms of staining factors such as pH, color, and solubility. Its ability to impart color can be attributed to the presence of anthocyanin and an acidic pH, suggesting that it has a cation that binds with the anionic bacterial cell wall. However, the results suggest that, currently, the ethanolic extract cannot be used as an alternative to crystal violet as there was an incomplete uptake of the stain. One factor that may have affected the uptake of stain was the staining procedure used that was a standardized procedure in staining bacteria using synthetic dyes such as crystal violet. The ethanolic extract may require its own developed staining procedure [11] due to its chemical structure and compatibility with counterstains, which may be different from synthetic dyes [21].

Color Intensity. The color intensity checks the contrast between the specimen and the background since stains are used to enhance contrast in the microscopic image [3]. The intensity of the color contrast is correlated with the purity of the stain, thus affecting its staining efficacy [25]. A darker stain can provide better contrast between the stained specimen and the lightly-colored background.

The color intensity of the ethanolic extract was evaluated in comparison to the color intensity of crystal violet in staining Gram positive *S. aureus* and

Gram negative *E. coli*. For the simple staining of *S. aureus*, the ethanolic extract had lighter color compared to crystal violet in 11 out of 15 replicates. Equal color intensity between the ethanolic extract and crystal violet was observed in 4 replicates out of 15. For the simple staining of *E. coli* bacteria, 13 out of 15 replicates stained with ethanolic extract through Gram staining had a lighter color on bacteria compared to crystal violet whereas three out of 15 replicates stained with ethanolic extract had equal color intensity to crystal violet, as in Figure 3.

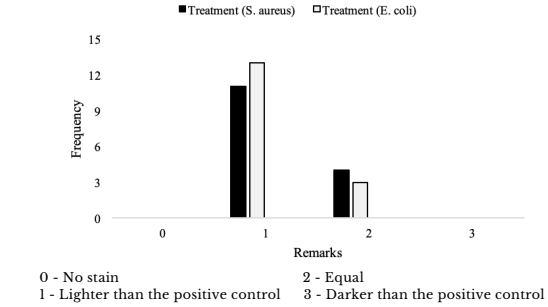


Figure 3. Color intensity of *Clitoria ternatea* ethanolic extract on *S. aureus* and *E. coli* through simple staining.

For the Gram staining, the color intensity was evaluated relative to the positive control. For *S. aureus* and mixture of the two bacterial smears, the color intensity of the ethanolic extract was evaluated based on the visibility and intensity of smears stained with crystal violet while the *E. coli* bacterial smears were evaluated based on the absence of purple stain or the intensity of the counterstain. All replicates of *S. aureus* and mixture of the two bacterial smears were observed to exhibit lighter color compared to crystal violet while *E.coli* bacterial smears had an equal color intensity compared to the positive control of stained *E.coli* bacterial smears, as in Figure 4.

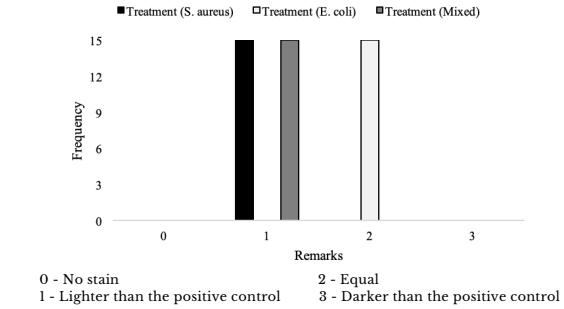


Figure 4. Color intensity of *Clitoria ternatea* ethanolic extract on *S. aureus, E. coli*, and mixture of the two bacteria through Gram staining.

Staining is performed to impart color to bacterial cells in order to highlight its morphological features [4] and to create contrast in the image viewed with the aid of a microscope [3]. Despite the ethanolic extract having a deeper shade of violet than crystal violet, it generally had a lighter color. This indicates that crystal violet, being a conventional biological stain [2], created a better contrast between the background and the bacterial cells compared to that of the ethanolic extract. The color intensity is affected by dye impurities [25], which may be present in the ethanolic extract. These impurities influence staining by altering the intensity of coloration imparted by the stain or by changing staining patterns, and the nature and staining mechanism [25]. The standardised staining procedure used that was designed for synthetic dyes may also attribute the lighter color imparted by the ethanolic extract on bacteria.

Limitations. Only three properties of the ethanolic extract were evaluated namely pH, color, and solubility. The test for the presence of anthocyanin was the only phytochemical screening done. The Pantone Colour Matching System, a standardized color reproduction system used in industries, was used due to unavailability of color charts for microbial stains. Due to the lack of standardized rubrics for evaluation, the researchers modified a rubric from Sridhara et al. [21] and had it verified by only one licensed medical technologist. The staining ability of the extract was also evaluated using one Gram-positive and one Gram-negative bacteria only.

Conclusion. Based on the findings of the study, the *Clitoria ternatea* ethanolic extract has the potential to stain Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* bacteria through simple staining. Also, it has the potential to stain and differentiate *S. aureus*, *E. coli* and mixture of the two bacteria using the Gram staining method. However, despite comparable results in the pH, color and solubility of the ethanolic extract and crystal violet, it cannot currently be used as an alternative bacterial stain because it was not able to stain the bacteria as effectively as the positive control in terms of the visibility of bacterial cell walls and color intensity.

Recommendations. The researchers recommend: (1) the development of a new staining technique for the potential stain; (2) the determination of other phytochemicals and functional groups present in the ethanolic extract; (3) the preparation of more oven-dried flowers to be soaked in order to accommodate more number of replicates per set-up; and (4) having varying concentrations for the potential stain.

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