Review Article

Staining Capability of Plant Extracts for the Identification of Gram-positive and Gram-negative Bacteria: A Systematic Review

Rae Antoinette D. Badar, Junissa Lalaine R. Carmona, John Gabriel C. Collantes, Daneery R. Lojo, Sean Mairose B. Ocampo, Roxette Lorie C. Ursua, Daniel H. Bercede*

Department of Medical Technology, Institute of Arts and Sciences, Far Eastern University Manila, Metro Manila, PHILIPPINES.

Submission Date: 26-05-2022; Revision Date: 19-07-2022; Accepted Date: 12-08-2022.

ABSTRACT

Background: Gram Staining is an essential diagnostic procedure where bacteria are stained to be properly identified. However, the stains used were found to be toxic with carcinogenic properties and (insert may) potentially harm living organisms as well as the environment. **Methods:** To address this issue, plant extracts were utilized in different experiments to test their staining capability on the bacteria. This study provides a systematic review of online articles and studies related to the staining capabilities of various plants. **Results:** Following the inclusion and exclusion criteria, a total of 137 papers were reviewed and only 11 papers were included in the study. Both the presence of phytochemicals and the pH concentration influenced the staining capacity of most plant extracts. In the following studies that were examined, most of the plants were able to stain gram-positive bacteria in comparison to the gram-negative bacteria. **Conclusion:** It was found that oxidized aqueous (hot or cold) extract of *Lawsonia inamis,* methanolic extracts of *Solanum melongena* L., 100% concentration of ethanolic extracts of *Ipomoea batatas*, ethanolic extract of *Clitoria ternatea*, *Pterocarpus osun* extracts, *Bixa orellana* extracts, and *Hibuscus sabderiffa* extract were able to stain the bacteria. Meanwhile, certain extracts have poor staining capability namely *Garcinia kola* mesocarp*, Vitex doniana* fruit*, Lantana aculaeta* fruit*, Cnestis ferrugnea* fruit*,* and *Pterocarpus soyauxii* stem. It is recommended to find other alternative extracts along with various extraction methods and concentration levels that may further enhance the affinity of the stain.

Keywords: Gram-positive bacteria, Gram-negative bacteria, Dyes, Stain, Plant extracts.

Correspondence:
Mr. Daniel H. Bercede, RMT, MSMT,
Department of Medical Technology, Institute of Health Sciences and Nursing, Far Eastern University Manila, Metro Manila, PHILIPPINES.

Email: dbercede@feu.edu.ph

INTRODUCTION

Gram staining is an important diagnostic technique that could determine various bacteria. It is a procedure that uses stains that can enhance certain characteristics of a bacteria to be visualized under the microscope and identify different types of organisms such as gram-positive and gram-negative bacteria. This method utilizes crystal violet and safranin red as primary stain and counterstain, respectively.

SCAN QR CODE TO VIEW ONLINE

www.ajbls.com

DOI: 10.5530/ajbls.2022.11.37

Crystal violet, also known as Gentian violet, is an organic chloride salt that is commonly used as a primary stain for gram stain in the identification of bacteria.[1] Anthocyanin, along with its alkaline chromogens, resembles the color of crystal violet stain.[2] They also described how the dissociation in the extract provides positive charges that can attach to the bacteria's negatively charged cell component. As a result, the stain can attach to the bacteria's cell wall. Furthermore, crystal violet is also recognized as an antiseptic for several infections concerning fungal, bacterial, and helminthic. It is also used as a dye for staining cloth, oil, and plastics, which are considered dyed waste products that are normally discarded into the environment, particularly in soil and bodies of water.

Safranin red, also known as safranin O or basic red 2, is also utilized as a stain for gram staining. Safranin

red distinguishes gram-negative and gram-positive bacteria by staining test organisms that didn't retain their primary dye or due to decolorization. Thus, it acts as a counterstain, aiding in the visualization and differentiation of bacteria. This stain enables the adherence of pink or red color to gram-negative cells by restricting acidic proteoglycans in body tissues and organs with high partiality.[3] Safranin red is considered a high-colored powder and water-soluble cationic dye, which is also used as a food coloring.[4]

Crystal violet and safranin red are synthetic substances that can potentially cause various health and environmental complications and pollution due to their chemicals, which could be toxic to living things. Crystal violet can continue its exposure in the environment and spread toxic chemicals which pose various health risks to exposed organisms. And due to the presence of carcinogenic properties and the ability to induce various health complications upon exposure to safranin red, its use is considered hazardous by the Occupational Safety and Health Administration.[5]

Phytochemicals are normally produced by plants and are often found in fruits and vegetables.[6] They are also beneficial to the overall antibacterial activity and color of fabrics.[7] Moreover, there are three major classes of phytochemicals, namely, carotenoids which give off yellow to red colors, chlorophyll which gives a green color, and lastly, anthocyanin which can give either blue, red, purple colors. Anthocyanin is part of the flavonoid group which is often found in berries, grapes, and other tropical fruits, but may also be found in vegetables like beets. In addition, stability and color changes of anthocyanin are susceptible to the pH of the solution which can give different colors in different pH conditions. For example, anthocyanin appears red when it is in acidic conditions, while for alkaline pH conditions, the color changes to blue. Therefore, anthocyanin is more stable in a lower pH solution.[8]

This review paper seeks to identify the best, natural, cheaper, and locally available dyes that can stain bacteria using gram stain. Moreover, this review looks for environmentally friendly dyes and least harmful to people compared to the standard dyes used. This can aid in the identification of the most innovative and efficient stain for identifying gram-positive and gram-negative bacteria. The researchers utilize the use of plants that can stain bacteria. Furthermore, this study also aims to provide the best alternative natural dye that is cheap and effective to aid small public hospitals or public schools that have tight budgets to conduct gram staining, which is the most used staining technique in the laboratory.

MATERIALS AND METHODS

Literature Search

Related studies were garnered from various credible search engines such as DOST SciNet-Phil, HERDIN, JK Prima, PubliScience, PubMed, ScienceDirect, Scribd, and Semantic Scholar. Search terms namely "Extracts", "Anthocyanin", "Gram Staining" and "Crystal Violet" were combined and utilized in the accumulation of studies without any restriction to allow researchers to collect literature that focuses on the staining capability of plants containing anthocyanin on gram-positive bacteria.

Selection Strategy

The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 schematic concept was used in this study, which is primarily designed for conducting systematic reviews and diagnostic accuracy statements. Before the full-text assessment, the title and abstract of each selected article were systematically evaluated by the researchers to confirm eligibility and discrepancies for inclusion in the systematic review. If consensus was attained, ineligible studies are deliberately excluded from the assessment and eligible studies proceed to the full-text review process.

Eligibility Criteria

This study is focused on the efficacy of plants with anthocyanin as cost-effective and environmental-friendly alternatives to crystal violet in staining gram-positive bacteria. The researchers selected credible articles that are related to the said topic and manually checked the abstracts to deduce the studies and come up with the following inclusion criteria: (1) Plant extracts that are used in gram staining technique of gram-positive and gram-negative bacteria, (2) Articles that utilized various extraction techniques in obtaining the plants' natural dye, and (3) Studies that are conducted from 2011 to 2022. While the exclusion criteria include: (1) Review and mini paper review, (2) Case reports, (3) Studies conducted earlier than 2010, (4) Studies from non-reliable sources, (5) Articles not written in English, and (6) Studies that are not related to gram staining.

Data Extraction

The researchers extracted the key information of the selected eligible studies including (1) name of the author, (2) year of publication, (3) abstract of the study, (4) plant identification, (5) test organism, (6) results and findings of the study. Any disagreements in the assessment process were settled through consensus. The data extraction was finalized by three authors (BRA, LD,

OSM), which was then reviewed and verified by three other authors for completeness and accuracy (CJL, CJG, URL). After completion of the included studies, Mr. Daniel Bercede acted as the final evaluator of the literature.

RESULTS

This section includes the study design wherein the gathered articles were critically chosen based on certain criteria. Furthermore, the results indicate a summary of the staining capacities of the plants and the effects of the different concentrations of the plant extracts on gram-positive and gram-negative bacteria based on the various studies involved. In Figure 1, a total of 152 published articles were retrieved using the specific terms during the initial search process. 15 articles were ruled out as duplicates and are, therefore, irrelevant to include for review. The following exclusion criteria are the basis for screening the 137 articles acquired: (1) studies not related to plant extracts, (2) studies not related to gram staining techniques, and (3) studies that are not related to gram-positive and gram-negative bacteria. As these reasons are contradictory to what the review is seeking to obtain, 109 articles classified under the mentioned criteria were excluded. Another set of criteria was used to identify eligible studies: (1) Review and mini paper review, (2) Case reports, (3) Studies that are outdated, (4) Studies from non-reliable sources, and (5) Articles not written in English. 17 studies that were distinguished from the criteria were eliminated and 11 studies were qualified for analysis.

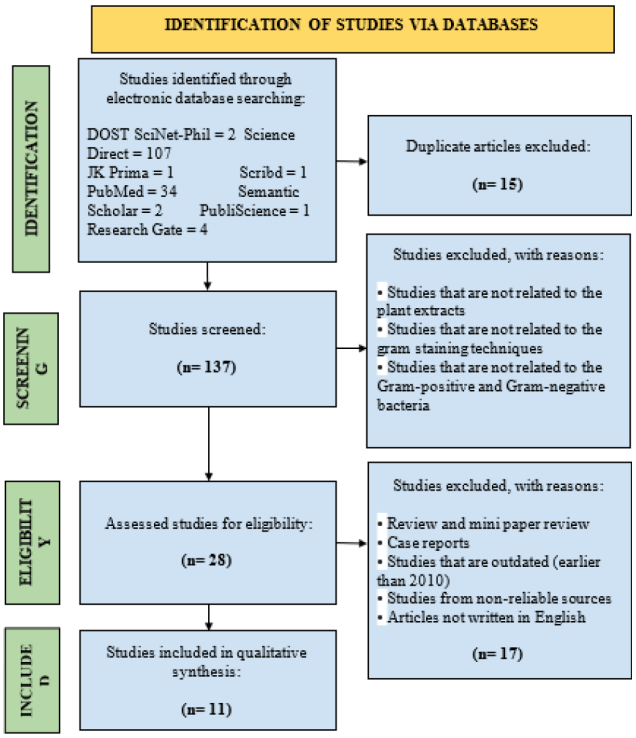

Figure 1: PRISMA 2020 Schematic Diagram depicting the study design process.

The Role of Phytochemical Compounds to the Staining Capacity of Plant Extracts

Generally, the classification of various phytoconstituents derived from plant extracts was significantly aided by phytochemical screening. Most phytocolorants include Anthocyanin, Carotenoids, Lawsone, and Daphniphylline which made significant contributions to various industrial applications such as dyestuffs and colorants. Based on the included articles shown in Table 1, eight out of eleven studies reported plants that contain anthocyanin compounds. These plant extracts include eggplant (*Solanum melongena* L.), Purple Sweet Potato (*Ipomoea batatas* L.), Blue Ternate (*Clitoria ternatea*), *Garcinia kola* mesocarp, *Vitex doniana* fruit, *Lantana aculaeta* fruit, *Cnestis ferruginea* fruit, *Pterocarpus soyauxii* stem, and Java plum (*Syzygium jambolanum).* Anthocyanins are naturally produced phenolic pigments that are generally regarded as the most significant unit of water-soluble dyes in nature, which are responsible for the phytoconstituent pigment such as the purple, red, orange and blue color of many plants.[9-11] Apart from this, there are three articles stated that the Henna plant (*Lawsonia inermis*) contains phytochemical pigment of Lawsone, a Hennotanic acid that responsible for red-orange pigment obtained from the leaves and two articles stated that Annatto (*Bixa orellana*) has bixin carotenoid that responsible for the red pigment. Moreover, the principal pigment of *Pterocarpus osun* is Santalins which are insoluble crystalline compound that are responsible for red pigment.[12]

The Effect of the pH Concentrations of the Extracts on Staining Capacity

pH is an important factor that may affect the staining ability of a dye. The colorization of the stain depends on various factors, including pH.[13] Changes in pH concentration influence the nature and the degree of charge on the components of the cell, thus altering the effectiveness of a dye to stain bacteria and other organisms. Out of the eleven articles analyzed, seven studies showed the effects of pH concentrations of the plant extracts on their ability to stain bacteria. The oxidized aqueous *Lawsonia inamis* with potassium permanganate showed to have a pH level of 7.00 to 7.16 having a better staining ability, while oxidized *L. inamis*

Table 1: Characteristics of included articles.

No.	Reference	Plant dye source	Extraction Medium	Test Organism	Phytochemical	Author's Conclusion
1	Francisco, *et al*., 2019	Eggplant peel (*Solanum melongena* L.)	Ethanolic extraction	*Escherichia coli* and *Staphylococcus aureus*	Anthocyanin component	
2	Chukwu, *et at*., 2011	Henna *(Lawsonia inamis* L.*)*	Aqueous (cold and hot) and ethanol extracts; alcoholic extraction	*Escherichia coli, Listeria monocytogenes, Staphylococcus aureus*	Lawsone (hennotannic acid)	The oxidized aqueous (hot or cold) extract of *Lawsonia inamis* has the potential to stain the test organisms (*Escherichia coli, Listeria monocytogenes, and Staphylococcus aureus) a*nd could be used as a substitute for counterstain in gram stain procedure.
3	Azucena, 2011	Eggplant peels (*Solanum melogena)*	Methanol extraction	*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Bacillus cereus*	Anthocyanin component	The *Solanum melongena* is feasible to use as an alternative for gram iodine but not as a primary stain.
4	Nunki, *et al*., 2020	Purple Sweet Potato *(Ipomoea batatas* L.*)*	Ethanol extraction	*Bacillus* spp.	Anthocyanin component	The *Ipomoea batatas* L. extract showed stain potentials and could be used as an alternative staining agent, but the study suggested further investigation and factor consideration regarding its effectivity to replace the Gentian violet in gram staining. The result recommended conducting a 100% extract concentration and 5 min staining duration.
5	Hafiz, *et. at*., 2012	Henna *(Lawsonia inamis* L.*)*	Aqueous and ethanol extraction	*Lactobacillus* spp. and *Escherichia coli*	Lawsone (hennotannic acid)	The oxidized aqueous (hot or cold) extract of *Lawsonia inamis* has the potential to stain the test organisms (*Lactobacillus* spp and *Escherichia coli*) and could be used as an alternative counterstain in gram staining.
6	Triol, *et al*., 2020	Blue Ternate (*Clitoria ternatea)*	Ethanolic extraction	*Staphylococcus aureus* and *Escherichia coli*	Anthocyanin component	The ethanolic extract of *Clitoria ternatea* can stain the *Staphylococcus aureus* and *Escherichia coli* through a simple staining procedure and gram staining. But the authors suggested developing of new staining method since the plant extracts are less effective than the positive control with regards to the color intensity and cell wall visibility.
7	Braide, *et al*., 2011	Zobo *(Hibuscus sabderiffa),* Uhe *(Pterocarpus osun),* Annato *(Bixa orellana*), Lalle (*Lawsonia inermis)*	A mixture of water, organic solvents (ethanol, absolute ethanol, methanol, chloroform), and aqueous solutions	*Aspergillus oryzae, Rhizopus stolonifer, Escherichia coli, Staphylococcus aureus*	*H. sabderiffa* (daphniphyllum-anthocyanin), *P. osun* (santalin A and B), *Bixa orellana (*bixin), *L.inermis* (Lawsone)	The application of acetic acid to plant extracts showed an increased staining ability to bacteria and moulds, while the use of ammonium hydroxide decreased their staining capability except for *Hibuscus sabderiffa* extract. The result concludes that the moulds stain better than bacteria. The study also showed that the *Pterocarpus osun* has the most favorable staining effect, while *Bixa orellana* and *H. sabderiffa* extracts have normal staining potential. The L*awsonia inermis* showed weak to no staining effect.

continued...

Table 1: Cont'd.

No.	Reference	Plant dye source	Extraction Medium	Test Organism	Phytochemical	Author's Conclusion
8	Enweani, *et al*., 2018	*G. kola* mesocarp, *V. doniana* fruit, *L. aculaeta* fruit, *L. inermis* leaf, *C. ferrugnea* fruit, *P. soyauxii* stem	Soxhlet solvent extraction technique using ethanol and methanol	*Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus mirabilis, Candida albicans, Aspergillus niger*	Flavonoids (Isoflavones and Anthocyanin)	The extracts have weaker staining capability compared to commonly used conventional synthetic stains due to the use of crude extracts in the procedure. The study suggests the use of a purer form of the plants' extracts for a better staining effect.
9	Comuelo, *et al*., 2020	Annatto seed (*Bixa orellana*)	Methanolic extraction	*Staphylococcus aureus* and *Escherichia coli*	Bixin (apocarotenoid)	*Bixa orellana* provided a colorless appearance after the bacteria was stained and are therefore not a good alternative for safranin in gram staining.
10	Aznar, *et al*., 2018	Java plum (*Syzygium jambolanum)*	95% ethyl alcohol solvent	*Bacillus subtilis* and *Escherichia coli*	Anthocyanin content	The use of *Syzygium jambolanum* extract is not a good alternative to crystal violet dye in staining *Bacillus subtilis* and *Escherichia coli.*
11	Hidayanti, *et al*., 2021	Purple sweet potato (*Ipomoea batatas L*.)	A mixture of ethanol, acetic acid, and water with a ratio of 25: 1: 5	*Staphylococcus aureus* and *Escherichia coli*	Anthocyanin content	The use of *Ipomoea batatas* extract with the concentrations of 60%, 70%, 80%, and 90% in staining gram-positive bacteria showed substandard results. The bacteria did not stain well as compared to using crystal violet dye. However, a 100% concentration of the extract was revealed to have a good staining capacity. On the other hand, all mentioned concentrations of the extract were able to stain gram-negative bacteria thoroughly.

ethanol extract with a pH level of 6.55 has no staining reaction.[14] A similar study also showed the same result for oxidized aqueous *L. inamis*, which suggest comparable staining affinity with safranin red.[15] The *C. ternatea* ethanolic extract and crystal violet have similar pH results with an acidic pH level value of 4.75 and 5.48, respectively.[13] Both are considered cationic stains that bind with the anionic bacterial cell walls, exhibiting presentable staining reactions. In addition to this, the staining ability of *P. osun* was decreased due to its high pH and the addition of ammonium hydroxide.[12] Six plants which are *G. kola, V. doniana, L. aculaeta, L. inermis, C. ferrugnea*, and *P. soyauxii* have pH levels ranging from 4.2 to 6.4 which are all acidic. The acidity in pH causes the extracts to have affinity for the cytoplasm to act as a counterstain.[16] *B. orellana* is also the extract used having an acidic pH level of 5.92, while the counterstain has a basic ph value of 7.44.[17] The result shows that *B. orellana* extract was unable to stain the bacteria in comparison to safranin. Moreover, the pH concentration of Java Plum extracts on *Escherichia coli* and *Staphylococcus aureus* as having the same results with crystal violet falling under acidic pH.[18]

The Difference between the Staining Capacity of the Plant Extracts to Gram-Positive and Gram-Negative Bacteria

Four studies assessed the difference in the staining capacity of the plant extracts between gram-positive and gram-negative bacteria. The results are variable based on the concentration of the plant extracts and whether alternatives for primary stain or counterstain are used. Both hot and cold aqueous extracts of the *L. inermis* oxidized with potassium permanganate proved to stain *E. coli* (gram-negative bacteria) effectively, even surpassing the staining capability of the positive control, counterstain.[15] Meanwhile, the extracts that were stained on *Lactobacillus* spp. (gram-positive bacteria) revealed no difference in the affinity of the control. Moreover, it was found that the acetone and methanol extracts of *B. orellana, P. osun, Hibuscus sabderiffa,* and lalle (*L. inermis*) plants are the most efficient mediums in staining moulds but not in bacteria.

[12] These extracts, when treated with glacial acetic acid and used as an alternative to counterstain, proved to stain *E. coli,* a gram-negative bacteria better than *S. aureus,* a gram-positive bacteria. This can be attributed to the characteristic of gram-positive bacteria retaining the primary stain due to the thickness of its peptidoglycan layer. The study also showed that the addition of ammonium hydroxide enhanced the staining capacity of the *H. sabderiffa* and *B. orellana* extracts only while it diminished the potential of the *P. osun* and *L. inermis* extracts to stain. Despite the increase or decrease in affinity, these extracts poorly stained both gram-positive and gram-negative bacteria. In another study, *S. aureus* is stained well using *P. soyauxii, L. inermis,* and *C. ferruginea* which can be comparable to the staining capacity of neutral red dye.[16] Conversely, all four plant extracts, *G. kola, L. inermis, C. ferruginea,* and *P. soyauxii,* stained gram-negative bacteria poorly. Therefore, these plant extracts are better alternatives to be used in counterstaining gram-positive bacteria than in gram-negative bacteria. Additionally, the gram-positive bacteria did not stain well in purple sweet potato extracts as a primary stain with concentrations of 60%, 70%, 80%, and 90%.[19] On the contrary, a 100% concentration of the said extract works best in staining gram-positive bacteria. In gram-negative bacteria, all concentrations have a good staining capacity. Based on these results, it is apparent that purple sweet potato extracts with concentration variability stain better in gram-negative bacteria than in gram-positive bacteria.

DISCUSSION

The presence of staining capability of plant extracts assists to enhance the features of gram-positive and gram-negative bacteria to be stained with gram staining. Considering the health and environmental harm that the conventional gram stains may induce, various plant extracts were used in separate experiments to test whether it can stain bacteria. Extraction processes differ for each plant and certain treatments were done to improve the affinity of the stain. In another perspective, there are differences in the staining capacity of plant extracts for gram-positive and gram-negative bacteria, which is based on what alternatives are used for primary stain or counterstain, therefore it led to having a variable result.[12,16,19] In this study, it is important to note what are the possible extracts that may or may not be successful in staining gram-positive bacteria and gram-negative bacteria to be an alternative natural dye for gram staining.

Staining of Gram-Positive Bacteria

Gram-positive bacteria stains purple as it is stained with crystal violet. It has a peptidoglycan layer which is thicker in comparison with gram-negative bacteria.[20] The findings of this systematic review showed that most of the plants can stain gram-positive bacteria.

Extracts obtained from the purple sweet potato peels came in various concentrations by combining appropriate values of the extract, ethanol, HCl, and NH_4OH. One study conducted saw that the dyes produced were alkaline dyes which also supports the idea that acidic elements shall be stained by basic dyes.[2] In addition, phenolic components of the gentian violet dye are also present in purple sweet potato peels. The natural dyes were able to stain the gram-positive bacteria, however, its staining capabilities are not on par with gentian violet stains. It is said that the increased pH affects the chromogenic factors present in the peels of the purple sweet potato. Another study utilized the use of purple sweet potato extracts and found that it is capable of staining bacteria.[19] The presence of anthocyanin in this plant produces a purple color when staining gram-positive bacteria using the most favorable concentration level. They propose to do further tests using the 100% concentration level extract and prolonging the staining time.

The methanolic extract obtained from *S. melongena* was able to stain various bacteria however, *Bacillus cereus* stained pink even though it is gram-positive bacteria which led them to conclude that the stain is not suitable for staining bacteria.[21] However, gram-positive bacteria may appear pink due to cell wall damage caused by antibiotic therapy or superfluous heat fixation, over decolonization of the smear, application of old mordant, or the smear came from an old culture.[22]

Furthermore, it was said that the ethanolic extract acquired from *C. ternatea* was capable of staining the *S. aureus* or a gram-positive bacteria through gram staining.[13] It has a low pH with a value of 4.75 which indicates a low and acidic pH. Plant extracts that have low and acidic pH signify cationic stains accompanied by good staining affinity for the anionic bacterial cell walls. However, it is suggested to still develop a new staining method considering that the plant extracts are less effective than the positive control in the matter of visuality of bacterial cell walls and color intensity.

Staining of Gram-Negative Bacteria

B. orellana seed, *L. Inamis* leaves, *H. sabderiffa* flowers, *G. kola mesocarp, V. doniana fruit, L. aculaeta fruit, C. ferrugnea fruit, L. inermis, P. soyauxii, and* heartwood of *P. osun*

were all extracted and utilized in staining gram-negative bacteria. The extracts mentioned acted as the Safranin dye or the secondary stain in the conventional gram staining procedure. All were unable to stain the bacteria without any form of treatment.[12-16]

With *L. inamis* extracts, 2 studies have concluded that potassium permanganate should be used in oxidizing the extract as it improves the staining of the gram-negative bacteria.[14-15] Oxidation of natural dyes, either by natural means or by the addition of chemical oxidants, was found to be more effective.[23] In addition, the said extract has a neutral pH which is a factor in attaining a better staining reaction. Acidic components have an affinity for basic dyes.[24] In the case of gram-negative bacteria, its cells are rich in nucleic acid which is an acidic component that explains why the oxidized extract is more effective as its pH is closer to a basic pH. Other chemicals used to modify the *L. inermis* extract, either as oxidants or accentuators, shifted the stain to be more acidic and gave unsatisfactory results.[14] The methanolic *B. orellana* seed extract also failed to stain the gram-negative bacteria due to its acidic pH of 5.92.[17] However, when treated with glacial acetic acid, *B. orellana* and *H. sabderiffa* were able to poorly stain gram-negative bacteria.

All the mentioned extracts were also unsuccessful in staining gram-negative bacteria when treated with ammonium hydroxide.[10] The addition of ammonium hydroxide has reduced the staining potential of the extracts. Ammonia can be used to clean stains.[25] The presence of ammonia in the ammonium hydroxide solution might explain why the treated stains performed poorly.

Interestingly, methanolic *P. osun* extracts treated with glacial acetic acid were able to stain gram-negative bacteria even though it was revealed that it is acidic.[9] It was discovered that *P. osun* extracts used as histological stains were able to stain in all pH conditions, but it decreased its staining capabilities in alkaline regions.[26] In addition, *G. kola, L. inermis, C. ferruginea, and P. soyauxii* alcoholic extracts were found to be acidic. However, it was able to poorly stain gram-negative bacteria. Researchers have said that this is due to the accentuator used as well as the chemical composition of each extract.[16]

CONCLUSION AND RECOMMENDATION

In conclusion, the extracted data presented in this systematic review provide conclusive evidence that some plants with the proper treatment have greater potential to replace the usual staining agents for the identification of gram-positive and gram-negative bacteria. About seven out of eleven studies proved that plant extracts can be used as organic staining agents for the identification of gram-positive and gram-negative bacteria. Extracts that were successful in staining bacteria with satisfactory results include oxidized aqueous (hot or cold) extract of *L. inamis,* methanolic extracts of *Solanum melongena L.,* 100% concentration of ethanolic extracts of *Ipomea batatas*, ethanolic extract of *C. ternatea*, *P. osun* extracts, *B. orellana* extracts, and *H. sabderiffa* extract. On the other hand, some extracts have poor staining capability namely *G. kola mesocarp, V. doniana fruit, L. aculaeta fruit, C. ferrugnea fruit,* and *P. soyauxii stem*. It was further observed that there are various factors, such as the level of pH concentration and choosing a solvent of extraction, that should be considered in producing alternative staining agents. Moreover, about two out of eleven studies showed some plant extracts, such as the ethanolic extracts of eggplant peels and methanolic extracts of *B. orellana* were not a viable substitute for the identification of gram-positive and gram-negative bacteria. However, its effectiveness as an alternative staining agent for gram staining technique needed further studies and development to fully establish the staining capability but the results showed potential. This paper recommends conducting further studies about the use of different extraction methods and their concentration to improve the staining ability of the plant extracts, and to determine the shelf-life of the mentioned potential stains. The oxidation of plant extracts to attain a pH level closer to basic pH is also suggested to enhance the staining ability in gram-negative bacteria. It also recommends finding other extracts that could be used as substitutes for commonly used gram stains.

ACKNOWLEDGEMENT

The researchers would like to extend their sincere gratitude to the Department of Medical Technology at Far Eastern University for imparting a great deal of knowledge, passion, and academic excellence to the researchers. To their research adviser, Mr. Daniel Bercede, and their research professors, Mr. Bernardino Hagosojos and Ms. Laarni Hannah Lacorte, for providing expert advice and various knowledge, and guidance throughout the journey. To their family, who show unconditional support and encouragement for the achievement of this study. And above all, to Almighty God for giving His wisdom, knowledge, and strength to make this review paper possible.

Author's Contributions

Authors B, C, and F wrote the introduction of the review which gave a brief overview of the topic and is composed of the important terms, discussion of problems, objectives and significance of the conducted analysis. Authors A, D, and E managed the methods which include where the list of articles was retrieved, standards for the qualification of the articles, and extraction of data. Author D and author E performed the selection strategy, PRISMA. All the authors collectively gathered the articles used in the study and proofread and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest

ABBREVIATIONS

DOST SciNet-Phil: Department of Science and Technology Science and Technology Information Network of the Philippines; **HCl:** Hydrogen Chloride; **NH_4OH:** Ammonium Hydroxide; **OSHA:** Occupational Safety and Health Administration; **pH:** Potential of Hydrogen; **PRISMA:** Preferred Reporting Items for Systematic Reviews and Meta-Analyses.

SUMMARY

This paper is a systematic review discussing the staining capability of various plants commonly found in the Philippines which were extracted for the identification of gram bacteria. Several factors affecting the staining effect of the plant extracts such as the phytochemical and pH concentration were discussed, including the different extraction mediums used to improve the staining of gram-positive and gram-negative bacteria. The findings revealed that *L. inamis, Solanum melongena* L., *Ipomoea batatas, C. ternatea, P. osun, B. orellana,* and *H. sabderiffa* extracts can successfully stain bacteria. Plants that are not mentioned have poor staining or needed further studies. Thus, this study showed that plants with proper extraction and pH level can replace the standard gram stains which may benefit the users environmentally and cost-effectively.

REFERENCES

1. National Center for Biotechnology Information. PubChem compound summary for CID 11057, Gentian Violet [cited May 4 2011]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Gentian-violet.
2. Nunki N, Titik Mutiarawati D, Prayekti E. Purple sweet potato (*Ipomoea batatas* L.) peels extract as an alternative dye for bacteria gram staining. IJMLST. 2020 August 27;2(2):76-84. doi: 10.33086/ijmlst.v2i2.1655.
3. Bishop J, McCue PM. Microbiology. Equine Reprod Proc. 2021:59-60.
4. Bensalah J, Habsaoui A, Dagdag O, Lebkiri A, Ismi I, Rifi EH, *et al*. Adsorption of a cationic dye (safranin) by artificial cationic resins Amberlite®IRC-50: Equilibrium, kinetic and thermodynamic study. Chem Data Coll. 2021;35:100756. doi: 10.1016/j.cdc.2021.100756.
5. ThermoFischer. Safety data sheet; 2014, August. Available from: https://imgcdn.mckesson.com/CumulusWeb/Click_and_learn/SDS_FSHSCI_PROTOCOL_SAFRANIN_500ML.pdf [cited 11/7/2022].
6. Thakur M, Singh K, Khedkar R. Phytochemicals: Extraction Process, safety assessment, toxicological evaluations, and regulatory issues. Science Direct. 2020 February 21:341-61.
7. Tiwari A, Srivastava M. Phytochemical analysis of selected natural dyes. International advanced research journal in science. Eng Technol. 2019;6(12):2394-1588.
8. Khoo HE, Azlan A, Tang ST, Lim SM. Anthocyanidins and anthocyanins: Colored pigments as food, pharmaceutical ingredients, and the potential health benefits. Food Nutr Res. 2017;61(1):1361779. doi: 10.1080/16546628.2017.1361779, PMID 28970777.
9. Martín J, Navas MJ, Jiménez-Moreno AM, Asuero AG. Anthocyanin pigments: Importance, sample preparation and extraction. Intech Open; 2017;15. p. 117-52.
10. Alappat B, Alappat J. Anthocyanin pigments: beyond aesthetics [internet]. MDPI. Multidisciplinary Digital Publishing Institute; 2020 [cited May 4 2022]. Available from: https://www.mdpi.com/1420-3049/25/23/5500/htm.
11. Francisco BMM, Garcia KMA, Geverola JPM, Kimilat CIL, Lagura ZMC, Lanojan DLT, *et al*. Anthocyanin in Eggplant peel (*Solanum melongena* L.) as Primary Stain in Gram-staining [internet]; 2019. Course Hero [cited May 4 2022]. Available from: https://www.coursehero.com/file/86884933/RESEARCH-PROPOSALdocx/.
12. Braide W, Akobundu C, Nwaoguikpe R, Njiribaeko LC. The use of extracts from four local Nigerian plants for the staining of selected bacteria and moulds. Afr J Microbiol Res. 2011 January 4;5(1):79-86.
13. Triol CB, Dionela ATV, Ecube AID, Mendiodia CJA. The use of *Clitoria ternatea* (blue ternate) ethanolic extract as a potential stain for bacteria. Publiscience. 2020;3(1):42-7.
14. Chukwu O. O. C. Application of extracts of henna (*Lawsonia inamis*) leaves as a counter stain. Afr J Microbiol Res. 2011;5(21):3351-6. doi: 10.5897/AJMR10.418.
15. Hafiz H, Chukwu OOC, Nura S. The potentials of henna (*Lawsonia inamis L.*) leaves extracts as counter stain in gram staining reaction. Bayero J Pure App Sci. 2013;5(2). doi: 10.4314/bajopas.v5i2.10.
16. Enweani IB, Olise NA, Oshim IO, Urama EU, Ngwoke CC. The use of extracts from Nigerian indigenous plants as staining techniques in bacteriology, mycology and histopathology. Int J Plant Res. 2018;8(1):15-24.
17. Comuelo JZ, Venturina SD, Samaniego WR, Jolito FC. The utilization of methanolic *Bixa orellana* (Annatto) seed extract as substitute for safranin in Gram-staining. Publiscience. 2020 June 25;3(1):33-6.
18. Aznar MSP, Manos MF, Ramos AJM, Yagonia TMC, Ymbong ML. The feasibility of *Syzygium Jambolanum* (Java Plum) as alternative for crystal violet in Gram-staining *Bacillus subtilis* and and *Escherichia coli* [internet]. Course Hero [cited May 4 2022]. Available from: https://www.coursehero.com/file/103578707/lib-researchdocx/.
19. Hidayanti ASN, Sulfiani S, Taufiq N. Utilization Pemanfaatan Ekstrak Kulit Ubi Jalar Ungu Sebagai Pengganti Crystal Violet pada Pewarnaan Gram. jsm. 2021;16(2):46-56. doi: 10.33761/jsm.v16i2.364.
20. Sizar O, Unakal CG. Gram positive bacteria. Stat. 2022 April 14. Cited in PubMed. PMID 29261915.
21. Azucena AG. Methanolic extract of eggplant (solanum Melogena) peel as a dye pigment in differential stains [internet]. Bartleby [cited May 4 2022]. Available from: https://www.bartleby.com/essay/Methanolic-Extract-of-Eggplant-Solanum-Melogena-Peel-F3CAL3SDJ3DQ.

22. Tankeshwar A. Variations in Gram-staining results. Microbe Online. 2021.
23. Carleton HM, Drury RAB, Wallington EA, Cameron R. Histological technique. 4th ed. Oxford: Oxford University Press; 1976. p. 108nnnnn.
24. Mescher AL. Junqueira's basic histology: Text and atlas. 14th ed. McGraw-Hill Education; 2018.
25. Bennett P. 10 Surprising ways to use ammonia around the house [internet]. Angi [cited Sep 22 2021]. Available from: https://www.angi.com/articles/9-weird-ways-windex-works-around-house.htm.
26. Avwioro OG, Aloamaka PC, Imosemi II. Staining of tissues with dye extracts of Pterocarpus Osun: Choice of a Fixative. Int J Morphol. 2005;23(4). doi: 10.4067/S0717-95022005000400019.

Cite this article: Badar RAD, Carmona JLR, Collantes JGC, Lojo DR, Ocampo SMB, Ursua RLC, Bercede DH. Staining Capability of Plant Extracts for the Identification of Gram-positive and Gram-negative Bacteria: A Systematic Review. Asian J Biol Life Sci. 2022;11(2):276-84.