5. Application of Ethanolic Plant Extracts as a Biological Stain

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Abstract

Most of the chemically synthesized stains pose a threat to environment and human health. Thus, the use of eco-friendly, renewable, safe, cheaper and non-toxic dyes as microbial stains have been described. The ethanolic plant extracts of various local dye yielding parts of plants: flowers of *Hibiscus rosa-sinensis*, *Clitoriaternatea* and *Butea monosperma*, stems of *Rubiatinctorium* and seeds of *Bixaorellana* has been experimented. The extracts at variable pH were utilized in simple staining of *Staphylococcus aureus* and *Escherichia coli* and the extracts of *L. inermis* and *C. ternatea* were used in Gram staining method. The staining ability of the extracts was determined in terms of visibility of cell wall and color intensity. The acidic natural stains showed better results for bacterial staining. The *C. ternatea* extract imparted efficient results than *L. inermis* extract; when used in Gram staining procedure. Thus, the present study has explored the possibility of using plant dyes as biological stains.

Keywords: Biological stains, ethanolic plant extracts, bacterial staining.

Introduction

A stain may be any colouring organic compound that, when combined with another substance, imparts a colour to that substance (Towet, 2020). Stains are generally used to add colour to animal tissues, plant tissues, microbes and spores to make them optically distinct and the technique is known as staining (Korade et al., 2014). Microorganisms viewed under the microscope need to be fixed and stained to improve visibility, emphasize morphological features,

and sometimes preserve them. In microbiology, this process is known as staining (Triol et al., 2020).

In the laboratory world, especially in the field of staining, microbiology is one of the keys to help provide information about the diagnosis of a disease. The existence of the development of staining procedures to assist in roughly observing the morphology of microorganisms helps in identifying parts of the cell structure of microorganisms and helps differentiate similar microorganisms. To study the properties and divide microorganisms into specific groups for diagnostic purposes, biological dyes and staining procedures with a light microscope have become the main tools in microbiology (Nunki et al., 2020).

Various types of staining techniques are used in microbiology such as simple staining, differential staining, gram staining, acid fast staining, endospore staining, etc. Colouration of microorganisms by applying single dye to a fixed smear is termed as simple staining. Basic dyes such as crystal violet, methylene blue and carbolfuchsin are most frequently used to determine the size, shape and arrangement of the cell. The differential staining procedure includes the application of more than one dye to distinguish organisms based on staining properties. The Gram's staining method is one of the most important differential staining method based on the ability of a cell to retain the crystal violet dye during solvent treatment, and on the difference in the microbial cell wall that is amplified. The cell walls for Gram- negative microorganisms have higher lipid content than Gram-positive cells (Chukwu et al., 2011).

Most stains in current use are chemically synthesized from cheap petroleum sources, show superior fastness properties, are widely available at an economical price and produce a wide variety of color. However, they cause skin allergies and other harm to the human body on exposure and produce toxic waste, also reducing soil fertility (Korade et al., 2014). However, they pose a threat to the environment and human health as some synthetic dyes contain allergenic components and toxic heavy metals, contributing to land, water and air pollution. 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 (Mani &Bharagava, 2016).

Remediation of this problem is the use of alternative dyes that are non-hazardous to living things. Research shows that extracts to produce dyes can be obtained from natural sources such as plants, animals, and soil. According to recent studies plant extracts are the best source for natural dyes. They hold the potential to serve as a cheaper stain (Adeyemo et al., 2017). Over 2000 dyes

are synthesized from various parts of more than 500 dye-yielding plant species, of which only about 150 have been commercially exploited (Korade et al., 2014). These natural dyes are known to be convenient, cheaper, safe, non-toxic, eco-friendly, renewable and biodegradable (Cheng et al., 2014).

The use of ethanol as the solvent during extraction optimizes anthocyanin extraction and is reported to perform better compared to using water as the solvent (Cheng et al., 2014). Flavylium cation is the basic chromophore of anthocyanin. It is electron deficient and highly reactive. The positive charge in its structure gives it the capacity to bind to the negatively charged bacterial cell walls, 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 (Saptarini et al., 2015). In staining, the pH of the stain affects the nature and degree of the charge on specific tissue structures, influencing the ability of the stain to adhere to it. It is known that tissue elements are attracted to the oppositely charged ions of the stain. Hence, it is necessary to lower the pH value of the natural stain with dilute HCl (Korade et al., 2014). Bacteria are generally stained better with cationic stains due to their anionic cell walls (Triol et al., 2020).

In the present study, six local dye yielding plants were chosen to obtain ethanolic extracts and their applications in bacterial staining were explored. Various dye yielding parts of the plant were used for the purpose namely flowers of *Hibiscus rosa-sinensis* (Hibiscus), *Clitoriaternatea* (Blue ternate) and *Butea monosperma* (Palash), stem of *Rubiatinctorum* (Madder), leaves of *Lawsoniainermis* (Heena) and seeds of *Bixaorellana* (Annatto).

Materials and Methods

Collection plant materials

Total six local dye yielding plants material including *H. rosa-sinensis*(flowers), *R. tinctorum* (stem), *B. orellana* (seeds), *L. inermis* (leaves), *B. monosperma* (flowers), *C. ternatea* (flowers) were collected from different sources of Akola district, Maharashtra. The plant material was sun dried for 10-12 days and then powdered with the help of grinder and weighed using weighing machine.

Extraction procedure

The powder of the six plant material was processed for ethanol extraction. For Heena, Madder and Annatto extract, 25gm of dried powder of each plant was dissolved in 100 ml of 50% ethanol as a solvent i.e. 1:4 in ratio. For Hibiscus and Blue ternate extract, 25 gm of dried powder

of each plant was dissolved in 150 ml of 50% ethanol i.e. 1:6 in ratio. For Palash extract, 25gm of dried powder of the plant was dissolved in 200ml of 50% ethanol i.e. 1:8 in ratio. All the plant extracts was then rotated on rotary shaker for 4 hours and then incubated overnight at 30°C and filter the extract prier shaking of 1 hr. Liquid extracts obtained were separated from the solid residue by filtration first by using muslin cloth and then by Whatman's filter paper no.42 The filtered extracts were the further concentrated by boiling for 15-20 minutes in boiling water bath and then cooled. The concentrated extracts are then kept in small sterile bottles and stored in refrigerator at 4°C for further use.

Assessment of physical and chemical properties

The properties of the extracts such as colour and pH were assessed.The colours of each of the ethanolic plant extract were noted and the initial pH values of the ethanolic extracts were measured using a pH meter.Afterwards, the pH of half of each plant extract was lowered using dilute HCl and maintained at pH 1.

Slide preparation

The clinical pure isolates of *E. coli* and *S. aureus* were obtained from the Microbiology Department, Shri R. L. T. College of Science, Akola. Thin Smears of *E. coli* and *S. aureus* were separately made on a clean grease-free glass side.

Staining of organisms with plant extracts

Using aseptic technique, bacterial smears were made on a clean glass slide, air dry and gently heat fixed.The slide was placed on a tripod stand and then flooded with the plant extract.Heat was provided with the help of burner till steam arises approximately for 5 minutes. Care was taken that the stain should not dry or boil on the slide.The slide was allowed to cool, and then rinse with deionized water until the water runs clear.Air dried and then examined under a light microscope.

Staining of organisms with plant extracts using Gram staining procedure

The bacterial smears were made on a clean glass slide and gently heat fixed. The ethanolic plant extract of Blue ternate was used as a primary stain in one case. In another case the ethanolic extract of Heena was used as a counter stain. The slides were examined under light microscope.

Result and Discussion

The large numbers of plants are dominated as a source of natural dyes, producing different colours. Six plant materials from local dye yielding plants are used in the present study.

Table 1 - Shows the Characteristics of the selected dye yielding plant materials.			
Sr. No.	**Scientific name**	**Common name**	**Part of plant used**
1.	*Rubiatinctorum*	Madder	Stem
2.	*Lawsoniainermis*	Heena	Leaves
3.	*Hibiscus rosa-sinensis*	Hibiscus	Flowers
4.	*Butea monosperma*	Palash	Flowers
5.	*Bixaorellana*	Annatto	Seeds
6.	*Clitoriaternatea*	Blue ternate	Flowers

The collected plant material was sun-dried for 10-12 days and then finely powdered by using the grinder. The weight of the dried powdered dyestuff was measured using weighing machine and noted (Table 2). The powder of the six plant material was processed for ethanolic extraction (50% ethanol).

The physical and chemical properties of the ethanolic extracts were assessed. The colour of the extracts was emphasized (Table 3). The pH values of the ethanolic plant extracts were measured using pH meter. According to the staining theory, the pH of the stain affects the nature and degree of the charge on the specific structures, influencing the ability of the stain to adhere to it (Triol et al., 2020). Hence, the pH of the extracts was lowered with dilute HCl and maintained at 1 (Table 4).

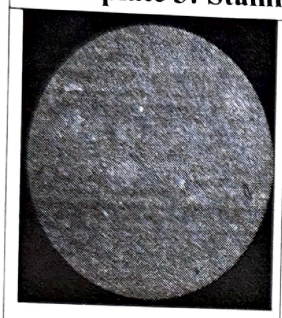
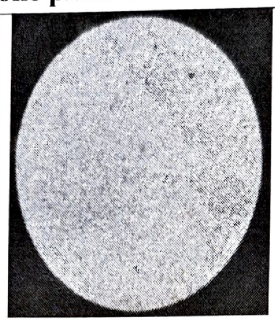
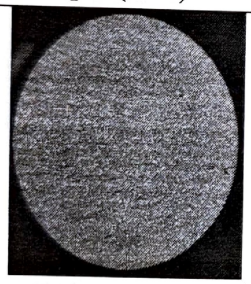
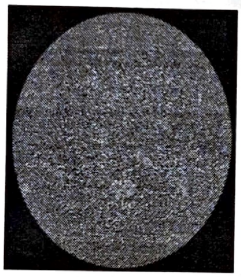
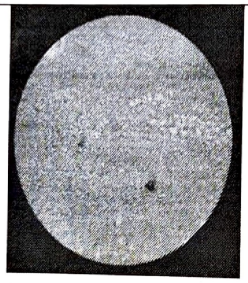
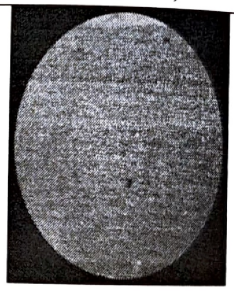
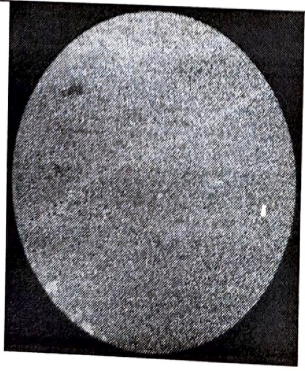
The clinical pure isolates of *E. coli* and *S. aureus* obtained from the P. G. Microbiology Department, Shri R. L. T. College of Science, Akola were confirmed by performing the Gram staining procedures. The colonies of *E. coli* were found to be Gram-negative, short rod and pinkish in colour. Whereas, the colonies of *S. aureus* were found to be Gram-positive in grapes like cluster with purple colour.

The smears of *E. coli* and *S. aureus* were prepared on grease-free glass slides. Each of the ethanolic plant extract was used for the staining of the thin bacterial smears and then subjected for microscopic examination. Each of the plant extract at its initial and final pH was subjected as a staining agent on both the bacteria. Both the bacteria were unable to stain with Madder and Heena at its initial pH, but found to be efficient at acidic pH. Due to the slightly acidic pH of Hibiscus, Palash, Annatto and Blue ternate extracts, bacteria was able to stain at both the pH. Since, more effective results was observed at final pH. The Madder stain imparted light red colour to the bacteria while the Heena gave green colour. Bacteria stained with Hibiscus were observed to be dark purple in colour. The Palash and Annatto imparted light yellow colour to the bacteria and was

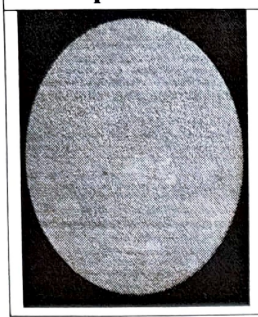
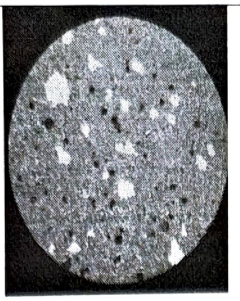
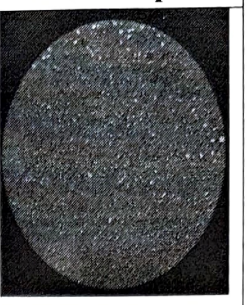
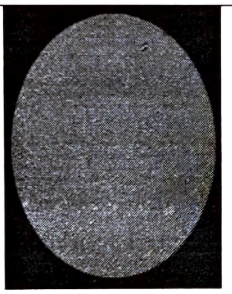
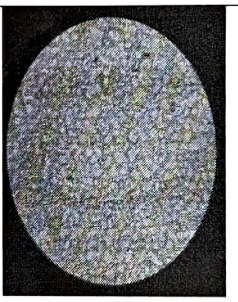
efficient and both the pH. The extract of Blue ternate have deep violet colour at its initial pH while it changes to bright pink under acidic conditions. The morphology of *E. coli* was observed to be short rods and that of *S. aureus* was cocci present in clusters.

Comparatively, *S. aureus* gave efficient results than that of *E. coli*. This may be attributed to the nature of its cell wall which is thicker than those of Gram-negative bacteria.

Photoplate 3: Staining of *E. coli* using ethanolic plant extracts at initial and final pH.

			
Photoplate 3.2: Madder at acidic pH (40X)	Photoplate 3.4: Heena at acidic pH (40X)	Photoplate3.6: Hibiscus at initial pH (40X)	Photoplate 3.8: Hibiscus at acidic pH (40X)
			
Photoplate 3.10: Palash at initial pH (40X)	Photoplate 3.12: Palash at acidic pH (40X)	Photoplate 3.14: Annatto at initial pH (40X)	Photoplate 3.16: Annatto at acidic pH (40X)
			
	Photoplate 3.17: Blue ternate at initial pH (40 X)	Photoplate 3.20: Blue ternate at acidic pH (40X)	

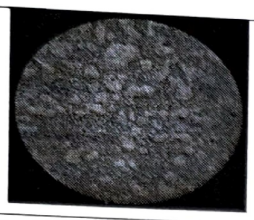
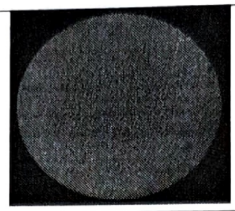
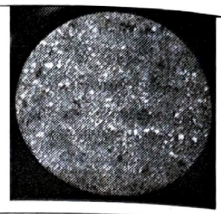
Photoplate 4: Staining of *S. aureus* using ethanolic plant extracts at initial and final pH.

			
Photoplate 4.2: Madder at acidic pH (40X)	Photoplate 4.4: Heena at acidic pH (40X)	Photoplate 4.6: Hibiscus at initial pH (40X)	Photoplate 4.8: Hibiscus at acidic pH (40X)
			
Photoplate 4.10: Palash at initial pH (40X)	Photoplate 4.12: Palash at acidic pH (40X)	Photoplate 4.14: Annatto at initial pH (40X)	Photoplate 4.16: Annatto at acidic pH (40X)
			
	Photoplate 4.18: Blue ternate at initial pH (40X)	Photoplate 4.20: Blue ternate at acidic pH (40X)	

The ethanolic plant extract of the *C. ternatea* (Blue ternate) when used as a primary stain in the Gram staining procedure, gave efficient results. The *E.coli* bacterial smears where evaluated based on the absence of the uptake of the primary purple stain or the intensity of the counterstain i.e. saffranin. They appeared as short rods with pink colour (Photoplate 5). *S.aureus* stained with the ethanolic extract had a defined cell wall and appeared to be cocci shaped present in clusters and were pinkish purple in colour (Photoplate7).

L. inermis (Heena) producing reddish brown dye was adapted as a counter stain in the Gram staining procedure. The morphology of *E. coli* was found to be short rods and were green in colour.

Since, it resulted in incomplete uptake of the stain, the bacterial cells were appeared to be faint in colour (Photoplate 6).*S. aureus* bacteria retain the primary stain, crystal violet with slight alteration in the intensity of the purple colour (Photoplate 8). It was observed that the staining reaction of the ethanolic Henna extract gives a better reaction with Gram negative bacteria *E. coli*.

			
Photoplate 5: *E. coli* stained with the ethanolic extract of Blue ternate using Gram staining.	Photoplate 6: *E. coli* stained with the ethanolic extract of Heena using Gram staining	Photoplate 7: *S.aureus* stained with ethanolic extracts of Blue ternate in Gram staining procedure.	Photoplate 8: *S. aureus* stained with ethanolic extracts of Heena in Gram staining procedure

Conclusion

The present study has explored the possibility of using plant dyes as biological stains in routine cytological works. The results showed that the ethanolic extracts of all the dye yielding plants can be considered as staining agents because it was able to impart colour to the bacterial cells. Based on the results, the stains at acidic pH were reported to improve their staining potential for bacteria. Having acidic pH values, the ethanolic plant extracts can be considered as Lewis acids, which make them cationic stains with good staining affinity towards the anionic bacterial cell wall. The ethanolic extract of *C. ternatea* (Blue ternate) can be a potential primary stain in the Gram staining procedure. As the Gram-positive bacteria *S. aureus* has ability to retain the primary stain after decolourization, it can be concluded that the stain taken by the bacteria was due to *C. ternatea* extract. Hence, it can be used as an alternative bacterial stain for crystal violet in the Gram staining procedure. Based on the finding, the Heena leaves ethanolic extract could not be used as a suitable substitute to the usual counter stain in Gram staining procedure. Since, it resulted in incomplete uptake of the stain due to very lighter appearance of the *E. coli*cells, also it interfered with the staining reaction of primary stain with *S. aureus* bacteria.

From the study final conclusion is,ethanolic extract of natural dye yielding plant materials can be employed as a staining agent that would be more convenient, cheaper, safe, non-toxic, eco-friendly, renewable and biodegradable.

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