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Development and Evaluation of Beetroot (Beta vulgaris L.) Root Extract as a Natural Staining Agent for Microscopic Bacterial Diagnosis

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ABSTRACT

Bacterial staining is an important procedure in microbiological examination; however, the routine use of conventional chemical stains raises concerns regarding human health and environmental safety. This experimental laboratory study investigated the preliminary microscopic staining potential of an aqueous Betalain extract obtained from *Beta vulgaris* as a natural alternative for staining environmental bacterial isolates. Aqueous extracts were prepared at concentrations 0.5%, 1%, and 3% and applied to heat-fixed smears of environmental bacterial isolates, including *Bacillus* and *Streptomyces*. Three independent replicates were performed for each concentration. Staining performance was evaluated using a semi-quantitative visual scoring system based on color retention, clarity of bacterial morphology, contrast between bacterial cells and the background, and resistance to stain loss during washing. samples treated with iodine as a mordant were compared with untreated samples. The results showed that staining intensity and microscopic contrast improved with increasing extract concentration, with the 3% extract providing the best overall staining performance. The use of iodine further enhanced dye retention and improved the visualization of bacterial morphological features. These findings indicate that aqueous *Beta vulgaris* extract, particularly when combined with iodine, demonstrates preliminary potential as an environmentally friendly natural stain for microscopic bacterial examination.

1. Introduction

The plant *Beta vulgaris*, commonly known as beetroot, is one of the most important root plants belonging to the Amaranthaceae family (Neelwarne, 2012). Beyond its nutritional value, it stands out in biochemical studies as a natural reservoir for phytochemicals (Clifford et al., 2015). The competitive advantage of this plant lies in its high content of unique nitrogenous pigments: betalains, which have become a focus for researchers as biological alternatives in pharmaceutical and diagnostic industries (Khan, 2016).

Betalains are water-soluble pigments, unique because they never coexist with anthocyanin pigments in the same plant (Stafford, 1994). Chemically, all betalain pigments share a basic structure called betalamic acid and are divided into two main classes: Betacyanins (red/violet, e.g., betanin) and Betaxanthins (yellow/ orange) (Gandía-Herrero & García-Carmona, 2013). These pigments contain functional groups such as carboxyl groups (***-COOH***) and amine groups (***-NH*** , ***=NH***), providing an ionic nature capable of reacting ***2*** with charges on living cell surfaces, making them excellent candidates for biological staining (Slimen et al., 2017).

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For decades microbiological laboratories have used coal-tar or petroleum-derived stains (e.g., crystal violet, safranin). These substances have been shown to possess mutagenic and carcinogenic properties (Littlefield et al., 1985), and their waste pollutes aquatic environments (Slama et al., 2021), despite their efficiency. This has resulted in the call for "Green Staining" through eco-friendly plant extracts (Riaz & Rashid, 2020).

1.1.Mechanism of Action and Cell Wall Interaction

To understand the mode of action of beetroot extract it is important to study its main target i.e. the bacterial cell wall. The structure is composed basically of peptidoglycan, a cross-linked network of alternating sugars and amino acids (Silhavy et al., 2010).

Gram - Positive Bacteria : Possess an unusually thick layer of peptidoglycan that contains teichoic acids. These impart strong negative electrostatic charges .

Gram-negative Bacteria: Smaller peptidoglycan layer but covered by another membrane with lipopolysaccharides (LPS).

The efficiency of any stain, including beetroot extract, relies on the ability of the molecules to penetrate or adhere to these structural layers. This interaction is due to hydrogen bonding or electrostatic interactions between the functional groups of the dye and the chemical constituents of the cell wall (Beveridge, 2001).

1.2.Application on Environmental Soil Isolates

The present study is of particular importance when applied to environmental isolates of bacteria from soil. Soil bacterial isolates, including representative strains from soil environments, are known for their complex cell walls and high ability for endospore formation (Madigan et al., 2020). The use of aqueous beetroot extract to stain these species is not just a matter of colorimetric aesthetics, but an assay of the chemical permeability of betalain pigments, assessing their capacity to penetrate these complex envelopes and bind to the peptidoglycan matrix (Yusuf et al., 2017).

1.3. Pigment Stability and Binding Affinity

In addition, it has been reported by researchers that the stability of beetroot extract is significantly affected by environmental factors such as light, temperature and pH (Azeredo, 2009). The best pigment stability was obtained at pH 4-5.

Other plant extracts were studied by Basu et al. (2015) . The results indicated that pigments with nitrogen atoms in their ring structures (such as betalains) are highly attractive to bacterial nucleic acids and proteins. Other studies have also been performed to study the feasibility of replacing safranin with natural red extracts. The results show that the extracts obtained from the plant produce enough contrast to observe the bacteria morphologies (bacilli and cocci) clearly at high power magnification.

2. Methodology

2.1 Aqueous Extraction

The extraction procedures was adapted from Azeredo (2009) with modifications.

Fresh Beta vulgaris L. roots were thoroughly washed, peeled, and cut into small pieces. A total of 1000 g of fresh beetroot was mixed with 1 L of distilled water (1:1, w/v) and heated for 1 h to facilitate pigment extraction. The mixture was then homogenized using a laboratory blender for 2 min and filtered through Whatman No. 1 filter paper. The filtrate was dried in a drying oven at 30°C for 3–4 h until a concentrated gel-like extract was obtained. The dried extract was stored at 4°C for 24 h prior to use.

A stock solution was prepared by dissolving 4 g of the dried extract in 100 mL of distilled water to obtain a 4% (w/v) solution. Working solutions of 3%, 1%, and 0.5% (w/v) were prepared immediately before staining by diluting the stock solution with distilled water according to the dilution equation ($C_1V_1 = C_2V_2$). For a final volume of 25 mL, 18.75 mL, 6.25 mL, and 3.125 mL of the stock solution were used to prepare the 3%, 1%, and 0.5%

solutions, respectively, with the remaining volume completed using distilled water.

2.2 Bacterial Culture and Smear Preparation

Environmental bacterial isolates obtained from soil samples were cultured in Nutrient Broth and incubated at 37°C for 18–24 h to obtain actively growing cultures suitable for microscopic staining (Cappuccino & Welsh, 2017). A loopful of a single bacterial colony was aseptically transferred into the culture medium for inoculation. Three independent biological replicates were prepared for each extract concentration. Bacterial smears were prepared on clean glass slides, air-dried, and heat-fixed by passing the slides through a Bunsen flame three times to ensure adequate cell adherence prior to staining (Cappuccino & Welsh, 2017).

2.3 Staining Protocol

The aqueous extract was used as a primary stain according to principles proposed by Basu et al. (2015). The slide was flooded with the extract for 3–5 min and gently rinsed with distilled water. For samples requiring a mordant, an iodine solution (Gram's iodine, comprising 1 g iodine, 2 g potassium iodide, and 300 mL distilled water; prepared in-house) was applied as a mordant for 1 min, followed by gentle rinsing with distilled water. Slides were then air-dried and examined under an oil immersion objective lens (100×, yielding a total magnification of 1000× using a Leica DM500 microscope equipped with a ICC50 HD digital camera).

2.4 Semi-Quantitative Evaluation of Staining Efficiency

Semi-Quantitative Evaluation of Staining Efficiency: Staining efficiency was evaluated independently by three experienced microbiologists using a single-blinded design to minimize bias.

The scoring was based on:

- Intensity of color retention
- Clarity of bacterial morphology
- Contrast between bacterial cells and background
- Resistance of stain loss during washing

Each evaluator scored the slides on a standardized scale, and any scoring disagreements were resolved through consensus discussion. Inter-rater reliability was assessed using Fleiss' kappa, yielding a high level of agreement among the evaluators.

Data Representation: Table 1 shows the semi-quantitative assessment of coloring efficiency.
The table (1) shows the semi-quantitative assessment of coloring efficiency.

Table 1: the semi-quantitative assessment of coloring efficiency

Score	Observation
+	Weak staining
++	Moderate staining
+++	Good staining

3. Results and discussion

3.1.Chemical Structure and Interactions of Betalain with Iodine

The major betalain pigment, specifically betanin (chemical formula: $\{C_{24}H_{24}N_2O_{13}$ (Strack et al., 2003), is characterized by the presence of hydroxyl (-OH) and carboxyl (-COOH) functional groups, conjugated double bonds (C=C), oxidizable phenolic moieties, and imine bonds (C=N). The nitrogen atoms in the imine groups bear lone pairs of electrons, representing the primary chromophore and active binding constituents of the broader betalain class.

Iodine can interact with these lone pairs on the nitrogen atoms but this is a weak and transitory interaction, not able to form either a stable salt complex or a durable long term covalent bond. Iodine does not form a stable salt complex but rather adsorbs on the pigment matrix or causes a partial oxidation of the functional groups in the betalain structure.

This phenomenon results in better colour stability and contrast on rinsing with iodine solution. Iodine enhances the aggregation of the betalain molecules, or decreases their solubility in water, so that the pigment is not easily eluted during washing of the slide.

So Iodine is a linking agent. It interacts with the lone pair of electrons on the nitrogen atoms of the betalain structure. It does not form a permanent covalent bond, but does allow for "entrapment" or adsorption, resulting in aggregation of the pigment molecules and a decrease in solubility in water during the washing phase (Basu et al., 2015). This will improve the sharpness of cell edges and the lifespan of the slide and we can show this in the table(1)

Table(1) Effect of *Beta vulgaris* (Beetroot) Extract Concentration on the Clarity of Bacterial Staining with and without Iodine Mordant.

Concentration of Root extract	Without Iodine	With Iodine Mordant
0.5%	Faint/pale bacilli; low contrast.	Slight improvement; still insufficient for diagnosis
1%	Moderate definition; short chains visible	Increased sharpness; clear morphological boundaries
3% (Raw)	High clarity; strong purple/pink contrast	Optimal quality; excellent contrast and stability

3.2. Results of bacterial staining:

3.2.1- Low Concentration (0.5%)

Without Iodine Treatment: The micrograph shows soil bacterial isolates stained with 0.5% aqueous beetroot extract. The contrast is poor and the bacilli sparsely distributed. The poor contrast prevents a precise separation of the bacterial cell wall from the surrounding background, which is due to the low density of betalain molecules at this particular concentration as showing in figure (1).

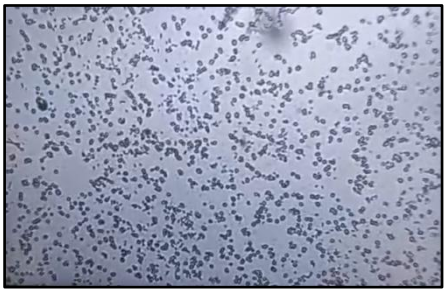

Figure (1): Microscopic appearance of bacterial isolates stained with 0.5% beetroot extract without iodine

With Iodine Treatment: This micrograph shows a slight improvement in clarity of the bacterial isolates. The iodine treatment helped to reduce the elution of the pigment during washing phase but the color density is still insufficient for precise microscopic diagnosis as showing infigure(2).

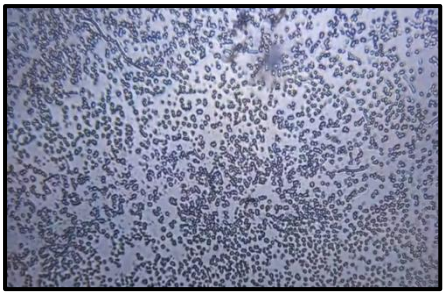

Figure (2): Microscopic appearance of bacterial isolates stained with 0.5% beetroot extract with iodine.

3.2.2- Intermediate Concentration (1%) Iodine is a linking agent. It interacts with the lone pairs of electrons present on the nitrogen atoms of the betalain structure. Though it does not form a permanent covalent bond, it induces 'entrapment' or adsorption which leads to the aggregation of pigment molecules and makes them less water-soluble during the washing step (Basu et al., 2015). This increases the sharpness of cell edges and the lifetime of the slide.

Without Iodine Treatment: This is a micrograph of the isolates of bacteria with moderate definition. The bacilli are clearly visible, and distributed and grouped in short chains. The good colour contrast enables the cell wall to be distinguished and thus demonstrates that the natural pigment is effective in binding to cell wall components in the initial stages as showing infigure(3).

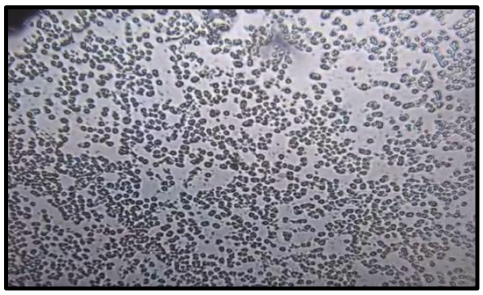

Figure (3): Microscopic appearance of bacterial isolates stained with 1% beetroot extract without iodine.

With Iodine Treatment: This micrograph exhibits a pronounced increase in the definition of bacterial morphologies. Iodine functioned as a mordant, enhancing the retention of the pigment within the pores of the cell wall matrix, thereby yielding a clearer image under the oil immersion objective lens as showing in figure(4).

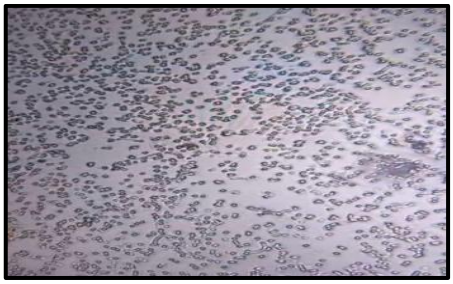

Figure (4): Microscopic appearance of bacterial isolates stained with 1% beetroot extract with iodine.

3.2.3- High Concentration (3% Crude Extract)

Without Iodine Treatment: This micrograph shows high definition visualization of the bacterial isolates . Dense distribution of cells into separate chains is visible. High contrast of color (intense purple/dark pink) is seen. This confirms the ability of the crude extract at this concentration to establish strong binding with the cell wall components of the environmental isolates even without any further chemical intervention as showing in figure(5).

Figure (5): Microscopic appearance of bacterial isolates stained with 3% beetroot extract without iodine.

With Iodine Treatment (Optimal Result):

The micrograph shows the best quality of staining. The bacilli and their cellular aggregations are more clearly visible and the contrast of the colour is optimal, so that the cells are completely separated from the background matrix. In this condition, iodine achieved the highest efficiency in stabilizing

the "betalain complex" in the cell wall framework, as shown in Figure 6

Figure (6): Microscopic appearance of bacterial isolates stained with 3% beetroot extract with iodine.

Based on the aforementioned results, the mechanistic role of iodine can be delineated as follows:

In the Absence of Iodine: The mechanism of staining is based on weak ionic interactions between the carboxyl groups of the betalain molecule and the peptidoglycan matrix of the cell wall. Thus, the pigment is less stable and can be easily lost during the water rinsing (decolorization) step.

In the Presence of Iodine: Iodine acts as a chemical coupling agent. The chromophoric properties are not affected but the dye molecules are trapped in the cell wall matrix by formation of a 'dye-iodine complex' which has limited diffusion through the structural pores of the bacterial cell wall (especially in Gram-positive soil bacteria) resulting in better definition of the cell boundaries. Superior color retention against the shear force of distilled water rinsing and prolonged lifespan of the microscopic slide prior to photobleaching.

3.3.Comparative Biochemical Analysis: Beetroot Extract vs. Safranin

A comparative biochemical discussion reveals a potential strategic advantage of the aqueous beetroot extract (Beta vulgaris) as a green, sustainable alternative to conventional synthetic stains such as safranin, based on theoretical efficacy in delineating bacterial morphologies. In terms of structure, the beetroot extract is a member of the natural nitrogenous betalain pigments that are based on betalamic acid, whereas safranin is a synthetic azo dye derived from complex aromatic petroleum compounds as documented in literature.

The difference in structure directly affects the kinetics of chemical binding with the bacterial cell wall. The main mechanism of action of safranin is the direct ionic attraction between the positively charged cations and the negatively charged cell surface. The molecule betanin (the main component of beetroot) is amphoteric in nature and possesses many polar functional groups, such as, carboxyl (-COOH) and amine (-NH2) groups. These functional moieties make it possible for betalain to generate a dense network of hydrogen bonds and electrostatic interactions with the peptidoglycan chains (Slimen et al., 2017).

While safranin gives a sharp and consistent contrast in crimson, the beetroot extract gives a warm contrast from purplish-red to dark pink under different conditions. This colouration provides excellent visual acuity and is very comfortable for long periods of microscopic examination (Azeredo, 2009).

From an environmental and occupational health perspective, synthetic stains such as safranin have been reported in literature to present potential health and ecological concerns (Slama et al., 2021). Alternatively, aqueous beetroot extracts derived from Beta vulgaris offer a more sustainable, plant-based alternative for laboratory applications (Riaz & Rashid, 2020), aligning with green chemistry principles due to their natural origin.

Table (2) Semi-Quantitative Evaluation of Beetroot Staining Efficiency

Concentration	Without Iodine	With Iodine	Morphological Clarity

0.5%	+	++	Weak visibility
1%	++	+++	Moderate visibility
3%	+++	++++	Excellent visibility

Results showed a concentration-dependent increase of the staining efficiency. The use of iodine greatly increased the retention of stain and cellular contrast.

The present study results showed that the aqueous beetroot extract with high contents of betalain pigments has promising staining potential for microscopic visualization of environmental bacterial isolates. Increased staining intensity and cellular contrast in a concentration-dependent manner was observed, which indicates the increased affinity of betalain molecules to bacterial cell wall structures.

The higher stainability at higher extract concentrations could be explained by the higher availability of betalain molecules that could form hydrogen bonds and electrostatic interactions with peptidoglycan components. Basu et al. (2015) reported similar results, emphasizing the suitability of nitrogen-containing natural pigments for microbial staining applications.

Also, iodine application noticeably improved color retention and morphological sharpness. It is hypothesized that iodine promotes the formation of transient interactions between betalain molecules and bacterial cell wall components, thus minimizing dye loss during washing steps.

This enhanced staining in the presumed Gram positive bacilli may be associated with the thick peptidoglycan layer of these organisms that allows the retention of dye in the matrix of the cell wall.

Beetroot extract had several environmental and biosafety advantages over synthetic stains like safranin, including biodegradability, low toxicity and reduced chemical hazards. Thus, betalain pigments from beetroot could be a sustainable alternative for educational and preliminary microbiological applicati

Limitations

This preliminary study has several limitations, including the absence of experimental standard-stain controls, pigment quantification, formal statistical analysis, definitive molecular isolate identification, and automated objective image analysis. These aspects should be addressed in future research to validate and expand upon these findings.

Future Recommendations

Limitations
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Future Recommendations
Future studies should incorporate standard synthetic stain controls, precise quantification of betalain pigments, rigorous statistical frameworks, molecular characterization of environmental isolates, and computerized image analysis tools to enhance reproducibility and clinical diagnostic accuracy**.**

4. Conclusions

The present study showed that aqueous beetroot extract containing betalain pigments can be used as a good natural staining agent for microscopic visualization of environmental bacterial isolates . The concentration of the extract and the iodine treatment significantly

improved the staining efficiency and stain retention, respectively, and cellular contrast.

The results support the potential use of beetroot extract as a low toxicity, biodegradable and environmentally sustainable alternative to conventional synthetic stains in microbiological laboratories, restricted to preliminary staining and educational applicability.

The study highlights the potential of betalain-rich beetroot extract as an eco-friendly biological stain and suggests the use of iodine-enhanced betalain interactions for better microscopic visualization of environmental bacterial isolates.

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