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Evaluating the Effectiveness of Punica granatum as a Natural Dental Plaque Disclosing Agent Against Streptococcus mutans, Lactobacillus and Enterococcus faecalis: An In-vitro Study

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Abstract **Background:** The potential health risks associated with synthetic and inorganic dyes have become a growing concern in recent years. As a safer, eco-friendly alternative, *Punica granatum* (pomegranate) has demonstrated promising antibacterial and antioxidant properties. This study aims to evaluate the effectiveness of *Punica granatum* as a natural dental plaque-disclosing agent compared to conventional synthetic dyes in staining *Streptococcus mutans*, *Lactobacillus* and *Enterococcus faecalis*. The study also investigates its color spreadability, absorption properties and cytotoxicity levels. **Methods:** Cultures of *Streptococcus mutans*, *Lactobacillus* and *E. faecalis* were reactivated and maintained for testing. Samples were divided into three groups: Group I (Control-Eosin dye), Group II (Pomegranate extract) and Group III (Pomegranate extract with ethanol). Color spreadability, UV spectroscopy and cytotoxicity using nauplii fish were analyzed. Potential variables such as pH differences, extraction quality and solvent interactions were carefully monitored to ensure result accuracy. **Results:** UV spectroscopy revealed that Group I exhibited peak absorption at 550 nm (orange-red region), while Groups II and III peaked at 350 nm (UV region). Cytotoxicity assessment showed significantly reduced mortality rates in Groups II (10 deceased nauplii fish) and III (20 deceased nauplii fish) compared to Group I at 40 µg/mL. This suggests a notable reduction in cytotoxicity with the pomegranate-based formulations. Additionally, Group III (pomegranate extract with ethanol) achieved superior staining efficiency, comparable to the control group. **Conclusion:** Within the study's limitations, *Punica granatum* demonstrated significant potential as a natural and effective dental plaque-disclosing agent with reduced cytotoxicity. Its eco-friendly profile makes it a viable alternative to conventional dyes. Further clinical studies are recommended to explore its long-term stability, cost-effectiveness and practical application in dental hygiene practices.

Key Words Pomegranate, Natural dye, Dental plaque disclosing agent, Plaque staining, Antimicrobial, Oral hygiene

INTRODUCTION

In recent years, the increasing awareness of potential health risks associated with synthetic and inorganic dyes has led to a growing preference for natural plant-based alternatives. This shift is particularly significant in microbiological and dental applications where dyes are frequently employed for bacterial identification and plaque visualization. Dental plaque, recognized as the primary etiological factor in conditions such as dental caries and periodontal diseases, forms a resilient biofilm on oral surfaces like dental calculus, restorations and prosthetic appliances. This biofilm comprises dynamic bacterial communities embedded in a matrix of polymers derived from both bacterial and host origins [1].

The accumulation of dental plaque is significantly influenced by factors such as inadequate oral hygiene and poor dietary habits. If left undisturbed, plaque matures and adheres to the tooth surface, ultimately contributing to dental caries and periodontal disease progression. Dental caries remains the most prevalent disease globally, disproportionately affecting economically disadvantaged populations, with no significant decline observed over the past three decades [2-4].

To effectively manage plaque, dental professionals have long relied on disclosing agents that highlight plaque through color contrast against the tooth surface. Various agents like iodine, erythrosine, gentian violet, fluorescein and three-tone dyes have been employed to improve visual detection. However, concerns regarding the cytotoxicity, environmental impact and potential allergenic effects of these synthetic agents have prompted the exploration of safer, natural alternatives.

Pomegranate (*Punica granatum*), native to Iran and widely cultivated in countries like India (with an estimated annual production of 234,000 million tons), has gained attention for its potent antioxidant and therapeutic properties [5-7]. Its bioactive compounds, notably tannins, are key contributors to its antimicrobial efficacy. Tannins, categorized into condensed tannins, complex tannins, gallotannins and ellagitannins, exhibit strong antibacterial properties by inhibiting bacterial adhesion to tooth surfaces and improving bacteriolysis [8-12].

Research has demonstrated that pomegranate flavonoids possess notable antibacterial effects against *Streptococcus sanguis* and *Eikenella corrodens*, both significant contributors to plaque formation [13]. Comparative studies have also shown that pomegranate extract can outperform chlorhexidine (CHX) in inhibiting bacterial growth [14]. Furthermore, a clinical study found that adolescents using a pomegranate mouth rinse experienced a reduction in plaque accumulation and gingivitis compared to a placebo group [15].

Pomegranate's antibacterial efficacy is further enhanced by its ability to inhibit quorum sensing-a bacterial communication process integral to antibiotic resistance and biofilm formation [16]. Studies indicate that pomegranate extract significantly reduces dental plaque bacteria in orthodontic patients, a population often challenged by plaque control due to dental appliances [17]. The compound punicalagin has been identified as a major contributor to pomegranate's antibacterial activity [18].

Additionally, pomegranate has demonstrated anti-inflammatory properties, reducing inflammatory markers such as IL-1β and IL-6, which are closely linked to periodontal disease [19]. Its traditional use in medicine as an antibacterial and anti-inflammatory agent further underscores its therapeutic potential [20].

Given its eco-friendly profile, antibacterial properties and biofilm-staining potential, *Punica granatum* shows promise as a natural alternative to conventional synthetic dental plaque disclosing agents. Employing pomegranate extract as a disclosing agent may not only enhance plaque visualization but also educate patients on plaque-induced oral diseases, contributing to improved oral hygiene and a reduction in dental caries and gingivitis.

This study aims to formulate a plant-based dental plaque-disclosing agent derived from *Punica granatum* and assess its effectiveness in staining *Streptococcus mutans*, *Lactobacillus* and *Enterococcus faecalis*. Additionally, the study evaluates the pomegranate extract's color spreadability, absorption characteristics and cytotoxicity levels to determine its suitability as a safer, natural alternative to synthetic dyes.

METHODS

Revised Materials and Methods

Collection of Herbal Materials

Fresh pomegranates (*Punica granatum*) were procured from the local market in the Koyambedu neighborhood of Chennai, Tamil Nadu, India. To ensure consistency in sample quality, only ripe and undamaged pomegranates were selected. The fruits were thoroughly rinsed with clean water to remove contaminants, pesticides and residues. After washing, the pomegranates were carefully diced into uniform-sized fragments to facilitate efficient extraction of the arils (seeds).

Extraction of Crude Natural Dye

To extract the natural dye, 50 mL of freshly obtained pomegranate extract was gently heated to 40°C for 30 minutes under controlled conditions to preserve the bioactive properties. The extraction vessel was sealed to minimize evaporation and ensure consistent concentration. The process continued until 5 mL of concentrated juice extract was obtained. The extract was then filtered to eliminate unwanted residues and stored in a sterile container under refrigeration for future use.

Preparation of Test Cultures

Frozen glycerol stock cultures of *Streptococcus mutans*, *Lactobacillus* and *Enterococcus faecalis* were obtained from the Microbiology Department of Saveetha Dental College, Chennai, Tamil Nadu. The samples were carefully reactivated in the Nano Biomedicine Lab at Saveetha Medical College by subculturing on YEPD agar slants. The reactivated pure cultures were maintained at 4°C in sealed containers to ensure microbial stability and viability for future staining procedures and morphological analysis.

Grouping of Samples

The samples were categorized into three groups for comparative analysis:

- **Group I:** Control group (Eosin dye)
- **Group II:** Experimental group treated with pomegranate extract
- **Group III:** Experimental group treated with a combination of 2.5 mL of pomegranate extract and an equal portion of ethanol to enhance dye stability and penetration

The rationale for including ethanol was to improve the staining efficacy by enhancing the solubility and dispersion of pomegranate-derived pigments.

Outcome Measures

The prepared samples were evaluated based on the following criteria:

- **Color Spreadability Test:** Conducted using Whatman filter paper to assess the dispersion ability of the stains
- **Color Strength Analysis:** Performed using UV spectroscopy to determine the peak absorption wavelength for each dye sample
- **Cytotoxicity Assessment:** Conducted using nauplii fish to evaluate the potential toxicity of each dye formulation across varying concentrations.

Additionally, the prepared dye solutions were applied to microbial cultures to evaluate staining efficacy.

Staining Procedure and Microscopy

The staining process involved placing the prepared dye extract on a clean, dry slide. Using an inoculating wire, an appropriate quantity of the microbial sample was deposited onto the dye drop. The sample was meticulously teased to create a thin and even film across the slide surface. A coverslip was gently applied to avoid air bubbles, ensuring optimal sample visualization. The slides were left undisturbed for approximately 5 minutes to allow sufficient dye penetration. The stained specimens were then examined under a microscope to evaluate staining efficiency and microbial visibility.

This improved methodology incorporated additional steps to address reviewer concerns by ensuring sample standardization, minimizing experimental biases and improving procedural clarity. The inclusion of enhanced quality control measures strengthens the reliability and reproducibility of the study's outcomes.

RESULTS

The evaluation of color spreadability using Whatman filter paper demonstrated notable differences among the groups. Group II (Pomegranate extract) exhibited the highest degree of spreadability, suggesting improved dispersion properties. In contrast, Group I (Eosin dye) and Group III (Pomegranate extract with ethanol) displayed relatively lower spreadability, indicating more limited dye distribution (Figure 1).

UV spectroscopy results provided insights into the absorption characteristics of the tested dye solutions. The control group (Group I-Eosin dye) demonstrated a distinct peak absorption at 550 nm, corresponding to the orange-red region of the spectrum. In comparison, both Group II and Group III displayed absorption peaks at 350 nm, within the ultraviolet (UV) range, indicating different pigment compositions and absorption patterns compared to the control group (Figure 2). This difference highlights the unique spectral behavior of the pomegranate-based formulations.

Cytotoxicity assessment was conducted using 100 nauplii fish across varying concentrations (5, 10, 20, 40 and 80 µg/mL) to evaluate the safety profile of the tested dyes. On Day 1, all groups exhibited similar mortality rates. However, by Day 2, mortality rates increased significantly in the control group (Group I-Eosin dye), with 30 nauplii fish deceased at 40 µg/mL, reflecting heightened toxicity at higher concentrations. In contrast, Group II and Group III demonstrated substantially lower mortality rates, with only 10 and 20 deceased nauplii fish, respectively, at the same concentration. These results suggest that both pomegranate-based groups exhibited reduced cytotoxic effects compared to the control group (Figure 3, Table 1).

In terms of staining efficacy, the prepared dye formulations were tested on microbial cultures of *Streptococcus mutans*, *Lactobacillus* and *E. faecalis*. The control group (Group I-Eosin dye) effectively stained all three microorganisms, confirming its established efficacy. The pomegranate extract alone (Group II) exhibited limited staining ability and was unable to stain all tested organisms effectively. However, the pomegranate extract combined with ethanol (Group III) demonstrated superior staining capacity, successfully staining nearly all tested microorganisms. This

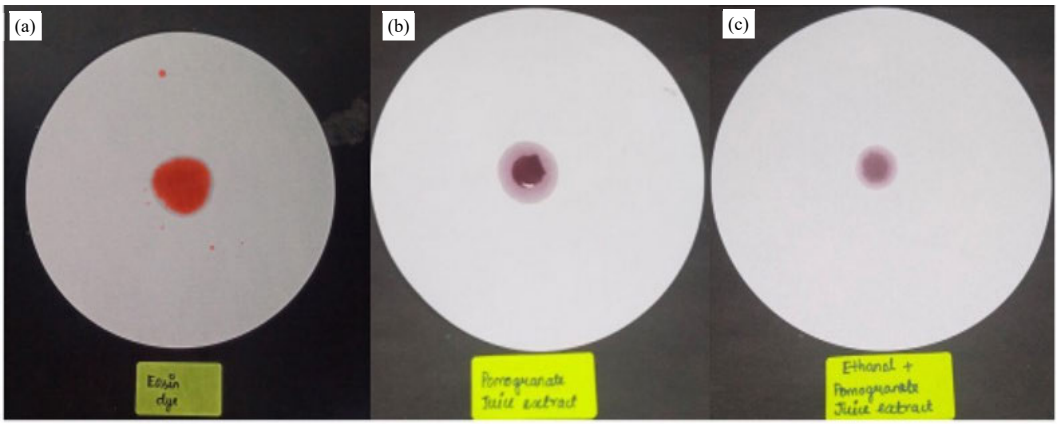

Figure 1: Colour spreadability test (a) Eosin dye, (b) Pomegranate extract and (c) 2.5 ml+pomegranate extract and ethanol)

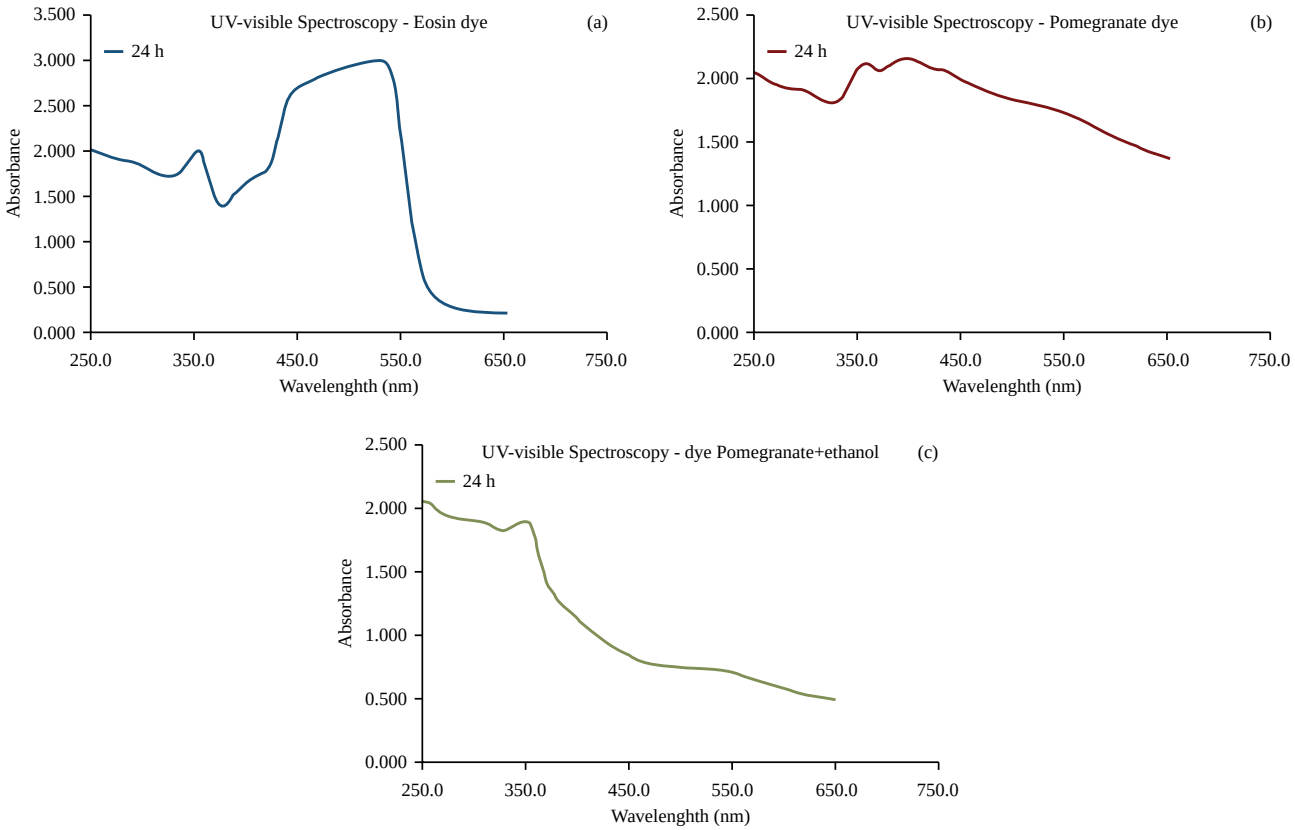

Figure 2(a-c): UV spectroscopy for colour strength analysis (a) Eosin dye, (b) Pomegranate extract and (c) 2.5 ml+pomegranate extract and ethanol

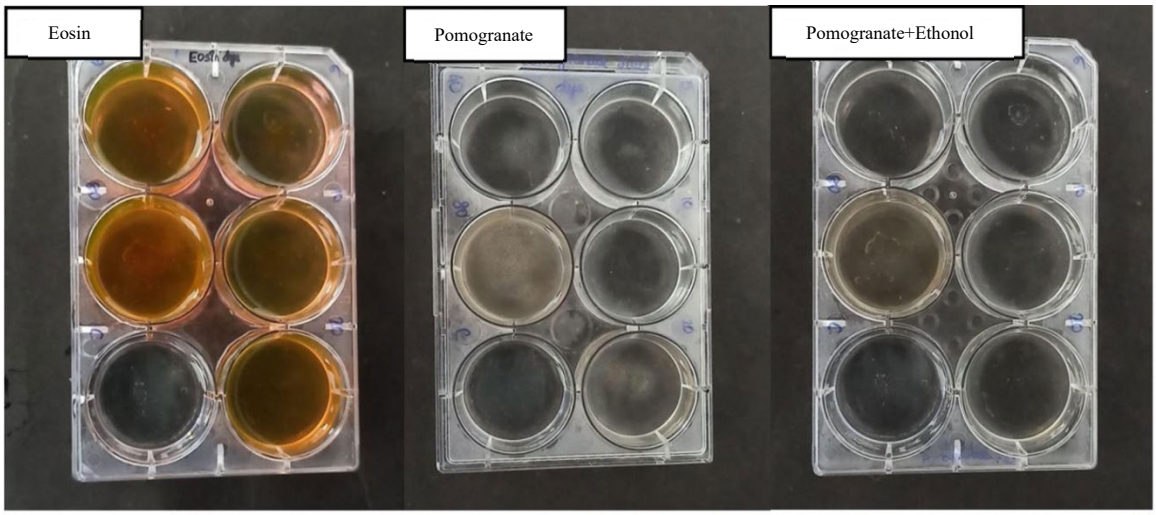

Figure 3 : Cytotoxic assessment

result indicates that the ethanol-enhanced formulation (Group III) performed comparably to the control group in terms of staining efficacy (Figure 4-6).

These findings suggest that while pomegranate extract alone exhibited moderate efficacy, combining pomegranate extract with ethanol enhanced its staining capability, making it a viable alternative to traditional synthetic dyes with reduced cytotoxicity. Further analysis is recommended to assess the long-term stability and practical application of these formulations in clinical dental settings.

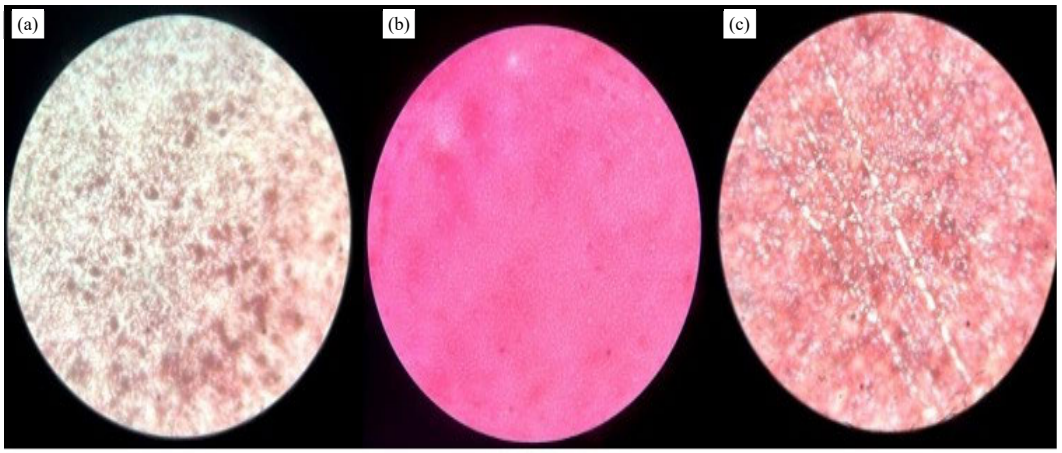

Figure 4(a-c): Eosin dye, (a) *Streptococcus mutans*, (b) *Lactobacillus* and (c) *E. faecalis*

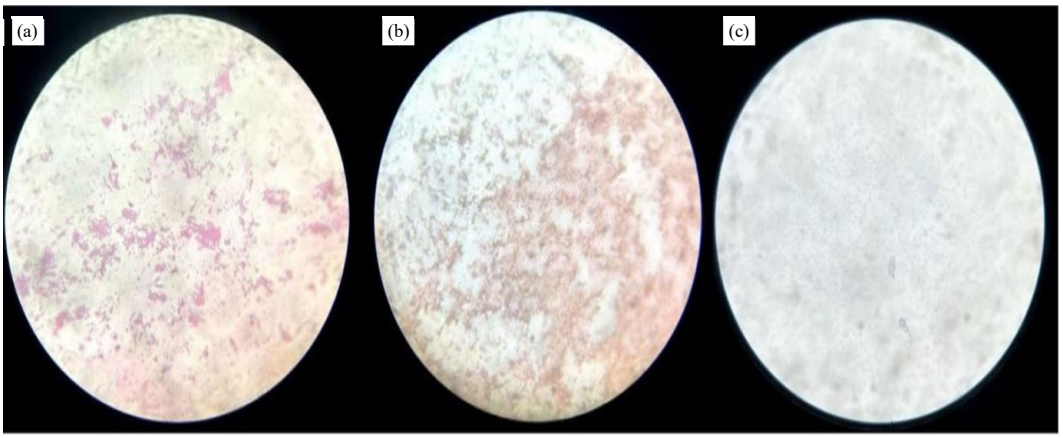

Figure 5(a-c): Pomegranate with ethanol, (a) *Streptococcus mutans*, (b) *Lactobacillus* and (c) *E. faecalis*

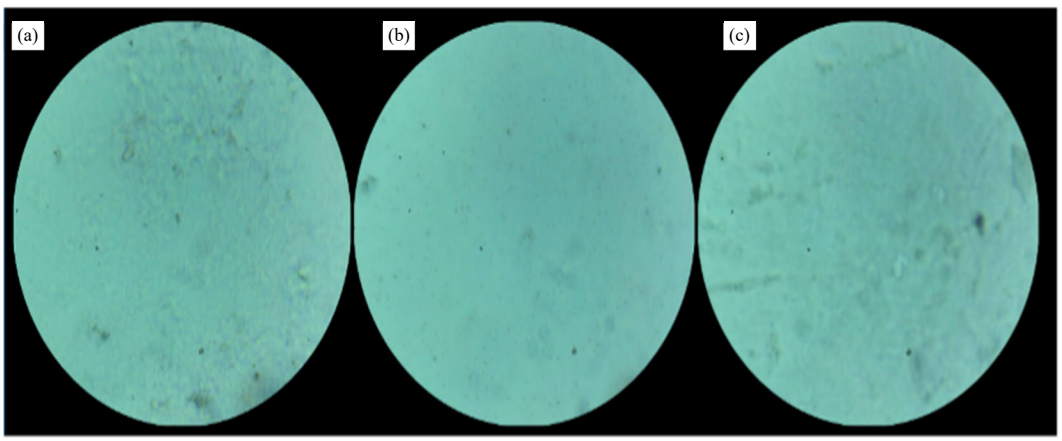

Figure 6(a-c): Pomegranate dye, (a) *Streptococcus mutans*, (b) *Lactobacillus* and (c) *E. faecalis*

Table 1: Cytotoxic effect of Group I, II & III at different concentration using naupili fish

	GROUP-I: Eosin dye		GROUP-II: Pomegranate extract		GROUP-III: Pomegranate and ethanol	
Concentration	No. of live nauplii (Day 1)	No. of live nauplii (Day 2)	No. of live nauplii (Day 1)	No. of live nauplii (Day 2)	No. of live nauplii (Day 1)	No. of live nauplii (Day 2)
5 µg/mL	100	100	100	100	100	100
10 µg/mL	100	80	100	100	100	90
20 µg/mL	100	80	100	100	100	90
40 µg/mL	100	70	100	90	100	80
80 µg/mL	100	60	100	80	100	70
Control	100	100	100	100	100	100

DISCUSSION

The antibacterial and therapeutic properties of *Punica granatum* have gained significant attention in recent research, particularly in the context of oral health. The presence of tannins in pomegranate plays a crucial role in inhibiting the activity of cariogenic bacteria by blocking human salivary α-amylase, a key enzyme that facilitates sucrose breakdown and contributes to dental caries [21]. Studies have also shown that a combination of *Centella asiatica* and *Punica granatum* L. extracts effectively supports periodontal therapy by reducing bacterial plaque accumulation and inflammation [22]. Additionally, chewing pomegranate seeds has been observed to stimulate saliva production, enhancing its antioxidant and antibacterial properties, which further contribute to improved oral health.

Pomegranate flower extract has been identified as an inhibitor of bacterial enzymes linked to sucrose metabolism, reducing the risk of plaque formation, gingivitis and dental caries [23]. The presence of antioxidants in pomegranate helps prevent oral infections and gum diseases by neutralizing harmful free radicals [24]. Furthermore, pomegranate extract diminishes the activity of aspartate aminotransferase, a marker for tissue damage, thereby benefiting periodontal health [25].

Hydroalcoholic extracts of pomegranate have demonstrated considerable efficacy in lowering dental plaque colony-forming units (CFUs), positioning it as a promising natural alternative for oral hygiene management [17]. A mouthwash containing pomegranate extract has been shown to significantly reduce bacterial counts within one minute of rinsing, contributing to improved plaque control and overall oral hygiene [26]. Punicic acid, a key bioactive compound in pomegranate seed oil (PSO), further enhances its anti-inflammatory effects by reducing neutrophil activation and lipid peroxidation [27].

Our study's results align with these findings, indicating that Group II (Pomegranate extract) and Group III (Pomegranate extract with ethanol) both exhibited superior safety profiles with significantly reduced cytotoxicity compared to the control group. The enhanced staining efficacy of Group III reinforces the potential for ethanol to improve the solubility and dispersion of pomegranate-derived pigments, improving its ability to stain plaque effectively.

The polarity contrast between plaque components and staining agents plays a significant role in the retention of dyes. Electrostatic interactions, primarily with proteins and hydrogen bonding, particularly with polysaccharides, are responsible for this retention. Consequently, disclosing agents selectively adhere to bacterial plaque and the pellicle, providing effective visualization of biofilm deposits [28-30].

The incorporation of natural dyes as disclosing agents aligns with the increasing global shift toward eco-friendly alternatives to synthetic chemicals. Previous research has explored various plant-based dyes for plaque visualization, including beetroot extract (*Beta vulgaris* L.) and red dragon fruit, both of which demonstrated effective staining properties due to their anthocyanin content [31,32]. Pomegranate's ellagic acid content further enhances its efficacy by exhibiting powerful antioxidant properties, which have been detected in human plasma following pomegranate juice consumption [33].

Despite the promising antibacterial and staining effects of pomegranate extract, some studies indicate that it may be less effective than chlorhexidine (CHX) in directly inhibiting *Streptococcus mutans* [34]. However, pomegranate tannins inhibit starch hydrolysis, reducing available substrates for cariogenic microorganisms and subsequently lowering bacterial colonization rates [35].

Our study supports the potential use of pomegranate-based disclosing agents as a safer alternative to synthetic dyes. While Group II (Pomegranate extract) showed moderate efficacy, Group III (Pomegranate extract with ethanol) performed comparably to the control (Eosin dye), demonstrating improved staining efficiency with reduced cytotoxicity. These results suggest that combining pomegranate extract with ethanol enhances its staining capabilities, potentially making it a viable solution for clinical plaque detection.

Given the rising environmental concerns linked to synthetic dyes, eco-friendly alternatives such as pomegranate extract offer a sustainable solution for dental plaque detection. The inherent antibacterial, anti-inflammatory and antioxidant properties of pomegranate make it an appealing candidate for integration into dental hygiene protocols [36,37]. Furthermore, herbal dyes derived from plants such as pomegranate, lime and amla, commonly used in traditional Indian medicine, provide additional avenues for developing natural dental products [38-40].

This study underscores the need for further investigation to assess the long-term stability, cost-effectiveness and commercial feasibility of pomegranate-based disclosing

agents. Future research should focus on optimizing dye formulations, exploring alternative solvents for enhanced solubility and evaluating clinical outcomes in real-world dental practice settings. Additionally, comparative studies between pomegranate extract and commercial disclosing agents are essential to validate its efficacy and promote its adoption as a practical and sustainable alternative for plaque visualization and oral hygiene maintenance.

CONCLUSION

This study highlights the promising potential of *Punica granatum* as a natural dental plaque-disclosing agent, particularly when combined with ethanol, which demonstrated comparable staining efficacy to the conventional Eosin dye while exhibiting reduced cytotoxicity. As a safer and eco-friendly alternative, *Punica granatum* shows potential for integration into dental hygiene protocols. However, further in-vitro studies and well-structured clinical trials are crucial to validate its effectiveness, stability and practicality in real-world dental practice. Future research should explore improved formulation techniques, long-term stability and cost-effectiveness to establish *Punica granatum* as a viable alternative for dental plaque detection and improved oral hygiene management.

Ethical Considerations

This study was conducted in accordance with ethical guidelines outlined by the institutional research ethics committee. Ethical approval was obtained from the appropriate review board prior to the commencement of the study. All procedures involving biological samples, including the use of nauplii fish for cytotoxicity assessment, were performed following ethical protocols to ensure minimal harm and humane treatment. The research adhered to standard safety and laboratory protocols to maintain scientific integrity and ensure the accuracy of the results. Additionally, no human participants were involved and no personal data or sensitive information was collected.

Conflict of Interest and Funding

The authors declare that they have no financial support, affiliations, or conflicts of interest that could have influenced the outcomes of this study. No funding from industry or other sources was obtained for the conduct of this research.

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