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Physicochemical and microbiological property of disclosing agent for biofilm detection: An *in-vitro* study

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ABSTRACT: This study aimed to evaluate the efficacy of the recently developed Ezy Gel D in identifying pathogenic characteristics of plaque, crucial for predicting the risk of caries. Distinct features of Ezy Gel D were explored, including its purple appearance and color transformation upon interaction with *Streptococcus mutans* biofilm. Acid production effects, viscosity, and pH stability were assessed over 60 min. Microbiological quality testing was conducted on day 1 and day 180. Ezy Gel D exhibited a notable purple-to-deep-blue color transformation interacting with *S. mutans* biofilm but showed no changes with acid production. The viscosity remained stable (low to medium), and pH values consistently ranged between 8.02 and 8.04. Microbiological testing on day 1 and day 180 revealed no issues, affirming product stability. The study confirms Ezy Gel D as a dependable and stable product for dental applications. Its favorable physical and chemical characteristics, coupled with the absence of microbiological issues, highlight its potential suitability for various dental procedures.

KEYWORDS: biofilm formation, caries, disclosing agent, dental plaque

INTRODUCTION

Dental plaque, an intricate amalgamation of microbial communities, extracellular polymeric substances, and host-derived components, constitutes a dynamic microcosm within the oral cavity [1]. This intricate ecosystem, with biofilm formation at its core, plays a pivotal role in oral health and disease. Understanding the nuances of biofilm development and its association with dental plaque is essential for unraveling the complexities of oral microbial ecology [2]. Biofilms, communities of microorganisms embedded within a self-produced matrix of extracellular polymeric substances, are ubiquitous in nature. In the oral environment, dental plaque serves as a quintessential biofilm, representing a sophisticated interplay between microbial colonization, host factors, and environmental dynamics. The ability of microorganisms to adhere to dental surfaces, coalesce into biofilms, and persist in this structured environment poses significant implications for oral health [3]. Moreover, the study on antibacterial and anti-caries compounds inhibiting bacterial growth, biofilm formation, and acid production gave rise to high potential preventive agents to cope for oral diseases [4].

The maintenance of optimal oral health hinges on the effective removal of dental plaque, a complex biofilm of bacteria that can lead to a spectrum of dental issues, including caries and periodontal disease. In the realm of preventive-dentistry disclosing agents have emerged as invaluable tools, offering a visual aid to enhance our understanding of plaque accumulation and improve oral hygiene practices [5].

Disclosing agents, typically formulated as tablets, solutions, or chewable compounds, contain dyes that selectively adhere to dental plaque, rendering it visible against the natural backdrop of tooth surfaces. This visual enhancement serves as a catalyst for heightening awareness and precision in plaque detection, thereby facilitating targeted interventions for improved oral health outcomes [6]. The use of disclosing agents, categorized into three groups, namely monotone staining, two-tone staining, and three-tone staining, is a prevalent technique in dentistry [7]. Monotone staining involves the application of a single color to aid in locating microbial deposits. The initial color commonly employed in dental practice is iodine solution, which serves to stain microbial deposits, enabling patients to visualize and effectively remove accumulations on tooth surfaces. While iodine has the advantage of being cost-effective, its drawbacks include reported allergic reactions and an unfavorable taste.

In addition to iodine, other monotone staining agents such as merbromin and neutral red have been explored. However, merbromin exhibited undesirable taste and staining characteristics, and its removal from the oral cavity proved challenging [8]. Moreover, neutral red led to red-colored urine in patients due to ingestion and renal excretion, prompting its discontinuation. Subsequently, there was a recommendation shifted towards the use of erythrosine, a dye certified by regulatory authorities for application in food, pharmaceuticals, and cosmetics, making it a preferred choice in dental practice [9].

Literature findings suggest the use of two-color staining agents in a dissolved form, which offers the advantage of clear and distinct staining of bacterial plaques on the tooth surface, especially in the interdental areas close to the gums. However, there are limitations to water-based product formulations, as mentioned earlier. In the present study, we utilized the two staining agents, erythrosine and brilliant blue, differing in molecular sizes and distinct colors of the stains. Then, a formulation, named 'Ezy Gel D' was developed from the two agents, which was capable of specifically marking areas that required staining and addressing issues of toothpaste smudging. This approach allowed the retention of colors in areas with varying levels of bacterial plaque density, resulting in clearer color patterns. This innovation aimed to generate interest in usage and contribute to testing the effectiveness of tooth brushing.

MATERIALS AND METHODS

Preparation of Ezy Gel D

Ezy Gel D, a toothpaste coloring gel, was prepared from P.C. Drug Center, Bangkok, Thailand, in a six-step process. Step 1: The Erythrosine and Brilliant blue dyes were meticulously weighed (4 mg and 1 mg), introduced into individual beakers, and completely dissolved into a uniform texture using a small amount of water (100 ml). Step 2: Sodium benzoate 0.1 mg, MC (Methylcellulose) 1 mg, and PVP K30 (Polyvinyl pyrrolidone) 0.5 mg were accurately weighed and added to their respective beaker. Step 3: A portion of water is dispersed, and PVP K30 is sprinkled to facilitate its dispersion in water. The contents of MC and PVP K30 were then combined as a solution and thoroughly mixed. Step 4: Sodium benzoate was added to the mixture in powder. Step 5: a homogeneous MC powder combined with PVP K30 was prepared by dispersing MC in hot water and stirring until a consistent texture is achieved. The final step, Step 6: the mixture from Step 4 was poured into the MC gel with continuous stirring until a clear gel was obtained. Then, flavoring, fragrance and 2 dyes in Step 1 were added, mixed thoroughly, and the final volume was adjusted to 100 ml. The culmination of these steps resulted in a two-color toothpaste coloring gel named Ezy Gel D product, specifically designed for staining bacterial plaques on the tooth surface.

Evaluation of the quality of Ezy Gel D

The quality of Ezy Gel D was evaluated, based on physical, microbiological, and product stability analyses, as follows:

Physical analysis: The analysis included visual appearance, opacity, and acidity-alkalinity levels.

Microbiological analysis: The analysis included total plate counts, yeast and mold levels, and detection of specific microorganisms including *Staphylococcus aureus*, *Candida albicans*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella* species, and *Clostridium* species.

Product stability testing is a critical aspect of pharmaceutical research and development, with the primary goal of verifying the effectiveness, quality, and safety of a given product. This comprehensive evaluation focuses on a long-term stability and its compliance to relevant standards. Key parameters examined included external appearance, color, acidity-alkalinity, opacity, and biological stability. Samples were systematically collected and subjected to extended environmental conditions in a climatic chamber set to a temperature of 30 °C ± 2 °C and a relative humidity (RH) of 75% ± 5%. This controlled environment was maintained for a duration of 6 months, allowing for a thorough assessment of the product's performance and attributes over an extended period. By scrutinizing these crucial factors, pharmaceutical researchers can gain valuable insights into the product's resilience, ensuring its reliability and suitability for use in various conditions.

Biofilm formation and acid production from *S. mutans*

S. mutans ATCC 25175 used for the testing was prepared by inoculating the strain from −80 °C and cultivating it on Brain Heart Infusion (BHI) agar. The BHI agar comprised a mixture of 29.6 g of BHI powder, 12 g of agar, and distilled water added to reach a volume of 800 ml in a 1,000 ml duran bottle, and the mixture was poured into plastic plates. The plates were then incubated at 37 °C for 48 h to obtain a single colony appearance. Following this, a 1 ml volume of the cultivated liquid medium of *S. mutans* ATCC 25175 was inoculated and incubated for an additional 18 h before testing. The obtained culture was then diluted in a solution of BHI and sucrose to achieve a bacterial concentration of 10^8 CFU/ml. Subsequently, serial dilutions were performed to obtain a bacterial concentration of 10^7 CFU/ml. The bacterial suspension was then tested in 96-well plates at various concentrations (repeating the dilution process three times). The bacterial cultures from each concentration group were incubated at 37 °C for 24 h. Afterward, the cultures were gently washed twice with distilled water (125 µl per wash), followed by the addition of Ezy Gel D. The negative control consisted of a colorless base solution [10].

For acid production method, *S. mutans* ATCC25175 strain, cultured in 100 ml BHI growth medium, was incubated in a shaking incubator at 130 rpm, 37 °C for 18 h, followed by centrifugation at 8,000 rpm, 4 °C for 30 min. The resulting cell pellet was resuspended in 7.5 ml of 135 mg/ml KCl solution; then, the cell suspension was adjusted to a concentration of 40 mg/ml and incubated at 37 °C for

Table 1 Physical properties of Ezy Gel D at day1 and day 180.

	Day 1	Day 180
Visual characteristic	Clear, viscous, purple liquid	Clear, viscous, purple liquid
pH ($n = 3$)	8.03 ± 0.01	7.23 ± 0.02
Turbidity ($n = 3$)	333.43 ± 0.22 cP	319.33 ± 6.03 cP

Table 2 Microbiological quality analysis of Ezy Gel D at day 1 and day 180.

Microbiological quality	Day 1	Day 180	SSV
Yeast and Mold Counts (CFU/ml)	< 10	< 10	< 10
S. aureus	NF	NF	NF
P. aeruginosa	NF	NF	NF
C. albicans	NF	NF	NF
E. coli	NF	NF	NF
Salmonella spp.	NF	NF	NF
Clostridium spp.	NF	NF	NF

NF = not found; SSV = standard specified values. Standard specified values are based on the testing criteria outlined in USP42/NF37; Microbiological Examination of Nonsterile Products: Oromucosal use.

3 h. The initial pH value of 7.0 was adjusted using a 0.02 mg/ml KCl solution for the negative control. Subsequently, 500 µl of 10% glucose was added. The culture was further incubated at 37 °C, and pH values were measured at 0, 30, 60, 90, 120, and 150 min. Ezy Gel D was applied during these time intervals, with a colorless base solution serving as the negative control [11].

Statistical analysis

All quantitative data were presented as mean ± SD. Qualitative data were described by characteristics such as color, excluding microbial count data. Physical property parameters were assessed using a one-sample t-test, with significance determined at p-values less than 0.05.

RESULTS

Physical property of Ezy Gel D

The results of Ezy Gel D's physical quality analysis, which included the product's visual color presentation, viscosity measurement, and pH assessment, were outlined in Table 1. Our investigation revealed that the Ezy Gel D product exhibited a distinctive purple gel appearance with a low to medium viscosity, falling within a stable range. The recorded pH values were consistently in the range of pH 8.02–pH 8.04, confirming the formula's stability. The pH and the turbidity of Ezy Gel D did not show a significant difference between day 1 and day 180. Additionally, microbiological quality testing of the Ezy Gel D tooth coloring gel on day 1 and day 180 did not reveal any microbiological issues, including *S. aureus*, *C. albicans*, *P. aeruginosa*, *E. coli*, *Salmonella* spp., and *Clostridium* spp. (Table 2).

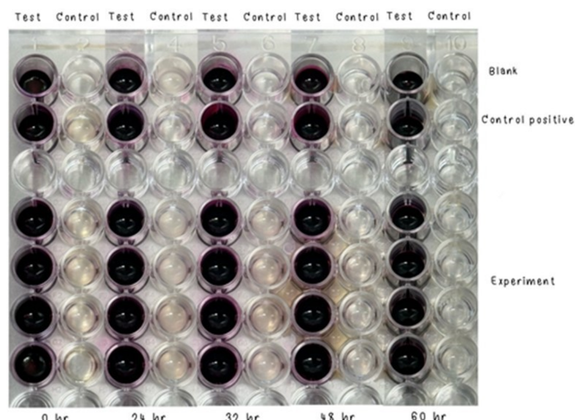

Fig. 1 Ezy gel D for biofilm formation *by S. mutans*.

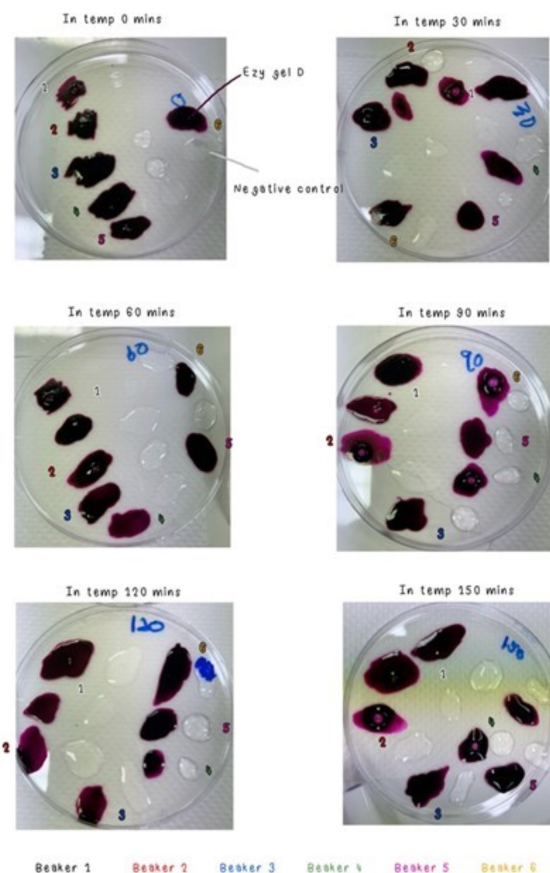

Fig. 2 Assessment of acid production by *S. mutans* after the application of Ezy gel D within the time range of 0–150 min.

Biofilm formation assay

The experimental group with biofilm from *S. mutans*, after a duration of 60 h, exhibited a color change from purple to deep blue; whereas the positive control remained a deep purple. Besides, there was a trans-

formation in the color of the base stain from yellow to clear, as shown in Fig. 1.

Ezy Gel D for acid production test

Comparing Ezy Gel D with distilled water (DW) and the positive control, it was observed thatEzy Gel D exhibited a deep purple color starting at 30 min until at 150 min. This indicates that Ezy Gel D could not be used to measure the acidity produced by *S. mutans* as shown in Fig. 2.

DISCUSSION

The associated risk factors encompass visible bacterial plaque and biofilm behavior, including plaque thickness, maturity, acid production, and levels of *S. mutans*. Furthermore, the presence of active caries and microbial loads in childhood significantly influences the oral environment, aligning with the principles of the ecological plaque hypothesis.

Now, with the advent of disclosing agents, the concept of visualization has become possible. These agents have been in use since the early 20th century, starting with the introduction of iodine by Skinner in 1914, followed by various organic solutions by Berwick in 1920 and Easlick in 1935, Merchurome solutions by Raybin in 1945, and King in 1951. Recent plaque disclosing agents are based on approved food colorants [12] such as basic fuchsin and erythrosine and their variants, as well as fluorescein disclosing agents and plaque probes [13].

Our investigation into the Ezy Gel D product revealed notable characteristics contributing to its overall quality and stability. The product exhibited a distinct purple gel appearance, which could not only serve an aesthetic purpose but also aid in easy identification. One crucial aspect of the product's performance was its viscosity, which was consistently measured within a low-to-medium range. Viscosity stability is essential for ensuring the product's ease of application and adherence to tooth surfaces, contributing to its effectiveness in various dental applications. The recorded pH values of Ezy Gel D, ranging consistently between pH 8.02 and pH 8.04, provided further evidence of the product's stability over time. Maintaining a stable pH is critical for dental products to ensure compatibility with oral tissues and to avoid potential adverse effects.

Microbiological quality testing conducted on the Ezy Gel D tooth coloring gel at both day 1 and day 180 did not reveal any microbiological issues. The absence of *S. aureus*, *C. albicans*, *P. aeruginosa*, *E. coli*, *Salmonella* spp., and *Clostridium* spp. underscored the product's adherence to high microbiological quality standards. This is particularly crucial in dental applications, where maintaining a sterile and safe environment is paramount, corresponding with a previously study report that dental disclosing solution was safe to gingival epithelial cells [13].

The present study explored the time-dependent color transformations exhibited by Ezy Gel D when exposed to biofilm from *S. mutans*, shedding light on its application in assessing plaque characteristics and acid production. After 60 h, Ezy Gel D demonstrated a vivid color shift from purple to deep blue when in contact with *S. mutans* biofilm. Surprisingly, when applied to acid production, no discernible color changes were observed.

Our findings revealed intriguing nuances in plaque behaviors. Newly formed plaque exhibited a sparse biofilm, allowing an easy removal of the blue pigment and resulting in a distinct pink/red coloration. In contrast, matured biofilm from old plaque ($>$ 48 h) displayed a dense structure that trapped both blue and red pigments, producing a characteristic blue/purple color [14, 15].

Moreover, in cases of extra high-risk plaque, the sucrose in the three-tone plaque disclosing gel (GC Tri Plaque ID Gel™) underwent metabolism by acidogenic bacteria within the plaque biofilm. This metabolic activity led to acid production, causing a reduction in plaque pH ($<$ pH 4.5) and the disappearance of the red pigment, resulting in a subtle light blue color [6, 14]. Ezy Gel D was primarily used in dental settings as a disclosing agent to highlight plaque and ensure thorough cleaning. Its gel-based formulation enabled precise application and visibility of plaque, typically applied with a brush or directly from the tube to the teeth. The gel adhered well to tooth surfaces, facilitating easy identification of plaque locations. Ezy Gel D stained plaque in distinct colors, often red or blue, pinpointing areas in need of additional cleaning. It offered a more even coverage, a better detailed staining, and particularly a less messy work compared with some liquid or tablet forms as it was less likely to drip or run [16]. The gel-like consistency of Ezy Gel D allowed for easy and even applications throughout the oral cavity.

Ezy Gel D showed stable pH and turbidity levels from day 1 to day 180, with no signs of microbiological contamination from pathogens such as *S. aureus*, *C. albicans*, *P. aeruginosa*, *E. coli*, *Salmonella* spp., and *Clostridium* spp. These results underscored its safety and efficacy, making Ezy Gel D a dependable option for applications like tooth coloring and other dental procedures where stability and contamination control are crucial.

Looking ahead, several potential directions could enhance its impact: conducting extended clinical trials to confirm long-term efficacy and safety across diverse patient populations; optimizing the formulation for better usability and color stability; exploring broader applications in orthodontics, cosmetic dentistry, and other medical fields; and performing additional microbiological studies to evaluate resistance to a wider range of microorganisms. Gathering feedbacks from

patients could also provide valuable insights for further refinements, while pursuing regulatory approvals and market expansion could improve accessibility and adoption. Known for its precision and effective staining, Ezy Gel D provided a clear visual representation of plaque, making it more effective than the sometimes messier or less controlled application methods of liquid-based agents or tablet systems.

Moreover, the findings of this study validated the association between caries, plaque, and cariogenic microorganisms. Caries status increased, as the mature and pathological scores did with *S. mutans* counts, illustrating the effectiveness of the three-tone plaque disclosing gel in identifying caries risk related to plaque. To substantiate these observations, additional longitudinal studies are essential, accounting for variables such as age and gender, and including assessments of anaerobic microorganisms like lactobacilli, which also contribute to caries progression. Given the rapid and observable nature of plaque pathogenicity, particularly with Ezy Gel D, it could be integrated into caries susceptibility testing for specific sites in a child's dentition known to be prone to carious lesions.

CONCLUSION

Our comprehensive assessment affirmed that Ezy Gel D possesses desirable physical and chemical characteristics, making it a reliable and stable product for dental applications. The absence of microbiological issues further supports its safety and quality, highlighting its potential suitability for a range of dental procedures.

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