ORIGINAL RESEARCH

Characterization of Cocoa Bean Husk Extract Particles and its Comparison as a Mouthrinse with Different Vehicles in Children aged 7–12 Years

Sabahath Kibriya[1], Ila Srinivasan[2], Jyothsna V Setty[3], Anu S[4], Bisma S Khan[5]

Abstract

Aim: Assessing the efficacy of cocoa bean husk extract (CBHE) particles with different vehicles as a mouthrinse in children aged 7–12 years in comparison to chlorhexidine (CHX) and sodium fluoride (NaF) mouthrinse.

Materials and methods: A total of 80 children aged 7–12 years residing at a residential school/orphanage in Bengaluru city were selected and randomly allocated into five groups—group I: 0.1% CBHE with distilled water (DW); group II: 0.1% CBHE with Ringer's lactate (RL); group III: 0.12% CHX; group IV: 0.1% CBHE with normal saline (NS); and group V: 0.05% NaF. A Simplified Oral Hygiene Index (OHI-S) was recorded, salivary pH was assessed, and unstimulated saliva samples were collected at baseline (BL) after 30 minutes of rinsing on day 7 and day 14. These saliva samples were subjected to microbiological analysis, and all the data from five groups at four different time intervals was tabulated and statistically evaluated.

Results: Nearly 0.1% CBHE with NS as vehicle showed maximum antibacterial properties among all the groups at all time intervals. The addition of RL to CBHE provided better anti-plaque efficacy than 0.1% CBHE with DW and 0.12% CHX mouthwash. All three combinations of 0.1% CBHE and 0.12% CHX mouthwash proved to be better anti-plaque agents than 0.05% NaF. Improving the preparation of CBHE mouthwash by using NS, RL, and with the addition of saccharin sodium also improved the patient's compliance.

Conclusion: Thus, preparing chocolate/CBHE mouthwash with NS or RL instead of plain DW increased the salivary pH, anti-plaque efficacy, and antibacterial property by reducing *Streptococcus mutans* growth.

Clinical significance: Cocoa bean husk extract (CBHE) mouthwash is a better anticariogenic and nonalcoholic mouthwash compared to CHX and NaF, which can be safely used in children as a routine oral rinse and also for those with gingivitis and high-risk of caries.

Keywords: Chocolate mouthwash, Cocoa bean husk extract mouthwash, Salivary pH, Sodium fluoride, *Streptococcus mutans*.

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Introduction

School-going children incline their dietary intake toward more fermentable carbohydrates and plaque-promoting substances, neglecting their oral health. As a result of this, it becomes difficult to prevent dental caries and gingivitis from time to time in their mouth. The majority of children brush their teeth once a day and very few practice flossing. Thus, oral rinses in children have been found to be one of the safest and effective vehicles[1] to disengage plaque from tooth surfaces inaccessible by brushing.

The most commonly used mouthrinse for children is CHX prescribed by dentists for gingivitis,[2] but it has certain disadvantages, such as altering the taste sensation, producing brown staining of teeth, affects mucous membranes, causing tongue sensitivity, and having an unpleasant taste.[1,3] Also the presence of alcohol makes it risky in younger children if swallowed in large quantities, thereby indicating the need for a safer alternative in the form of herbal polyphenol-rich preparations.

Cocoa bean husk (CBH) is part of the cocoa bean, separated from the cotyledons together with the germ during the preroasting or after the roasting process of *Theobroma cacao*.[4] Several studies about cocoa beans suggested that after its fermentation, CBH may be an important source of bioactive compounds, such as theobromine and phenols, which, together with the high fiber content imparts antibacterial/anti-glucosyltransferase (GTF) activity that inhibits biofilm formation and acid production by

[1,2,4,5]Department of Pediatric Dentistry, Mathrusri Ramabai Ambedkar Dental College & Hospital, Bengaluru, Karnataka, India

[3]Department of Pediatric Dentistry, Mathrusri Ramabai Ambedkar Dental College & Hospital, Bengaluru, Karnataka, India

Corresponding Author: Sabahath Kibriya, Department of Pediatric Dentistry, Mathrusri Ramabai Ambedkar Dental College & Hospital, Bengaluru, Karnataka, India, Phone: +91 9902291385, e-mail: sabahathk9@gmail.com

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S. *mutans* at a concentration of 1 mg/mL in DW.[5,6] This preparation, in comparison with the gold standard (CHX), proved efficient, but its bitter aftertaste and poor shelf life hindered its clinical use, thus leading us to explore firstly its particle characterization before preparing mouthwash, followed by replacing the vehicle media with NS or RL, and using a non-cariogenic sweetener to improve its taste. Thus, in the present study, we evaluated and compared the efficacy of CBHE with different vehicles as a mouthrinse against gold standard mouthrinses in children aged 7–12 years.

Materials and Methods

Study Design

A clinical-based interventional and microbiological study was conducted in children belonging to the age-group of 7–12 years (mixed dentition) residing at a residential school/orphanage. The ethical approval for the study was obtained from the Institutional Review Board and Ethical Committee (EC 649). A sample size of 80 was selected and randomly allocated into five groups, each comprising 16 subjects based on inclusion and exclusion criteria.

The five groups were as follows (Figs 1 and 2):

- Group I: Nearly 0.1% CBHE with DW mouthrinse.
- Group II: Nearly 0.1% CBHE with RL mouthrinse.
- Group III: Nearly 0.12% CHX mouthrinse.
- Group IV: Nearly 0.1% CBHE with NS mouthrinse.
- Group V: Nearly 0.05% NaF mouthrinse.

Inclusion Criteria

- Patients belonging to the age-group of 7–12 years.
- Patients having positive/definitely positive Frankl's behavior.[7]
- Patients having DEF/DMFT = 0 or <6.[7]
- Patients having a mild/moderate OHI status.[8]

Exclusion Criteria

- Medically compromised children, children with special care needs, and those with intellectual disabilities.[7,9]
- Patients undergoing any surgical treatment[7] or who recently underwent topical fluoride application.[9]
- Patients with a history of antibiotic therapy since the last 2 months.[10]
- Patients having a history of allergy to any of the components of mouthrinse used.[9]
- Patients whose parents/guardians/heads of the institution have not consented for permission.

Characterization of CBH Particle

Cocoa bean husk (CBH) (raw material) was washed, subjected to prolonged drying to remove moisture, and grinded into fine powder. The desiccated powder material was extracted using 100 mL of solvents *viz.* methanol by means of Soxhlet apparatus for 36 hours. The extract was then lyophilized to powdered form which was further subjected to the Olympus IX71 microscope, spectrophotometer for the estimation of particle size and surface roughness (range of 1000–100*X). A standard solution of the powder was made with deionized water, injected in triplicate, and analyzed using gas chromatography–mass spectrometry (GC-MS).

Preparation of CBHE

- A total of 1 kg of CBH were first treated with 5 gm of cellulose in 4.75 L of DW at 50°C for 4 hours.
- Ethanol was then added up to 50% (v/v final concentration), and the mixture was refluxed for 1 hour.
- After filtration, the ethanol was removed by evaporation and the aqueous solution was lyophilized to produce a powder. This process yielded 120 gm of powdered extract.
- The powder was dissolved in DW/NS/RL to obtain a mouthrinse with a final concentration of 1 mg/mL in 0.1%. Around 0.8 gm/L of saccharin sodium was added to all the CBHE mouthwashes as an artificial sweetener

Clinical and Microbiological Part

Subjects in each group were scored for plaque using OHI-S/OHI-S modified index,[8] salivary pH was assessed, and unstimulated saliva samples were collected. Children were instructed to use their respective mouthrinses twice daily for 2 weeks, using 10 mL for 30 seconds[7,10] in each session.

On day 1, 30 minutes[8] postmouthrinsing, another unstimulated saliva sample was collected, OHIs (OHI scores) and pH were determined. Furthermore, plaque score and change in pH was

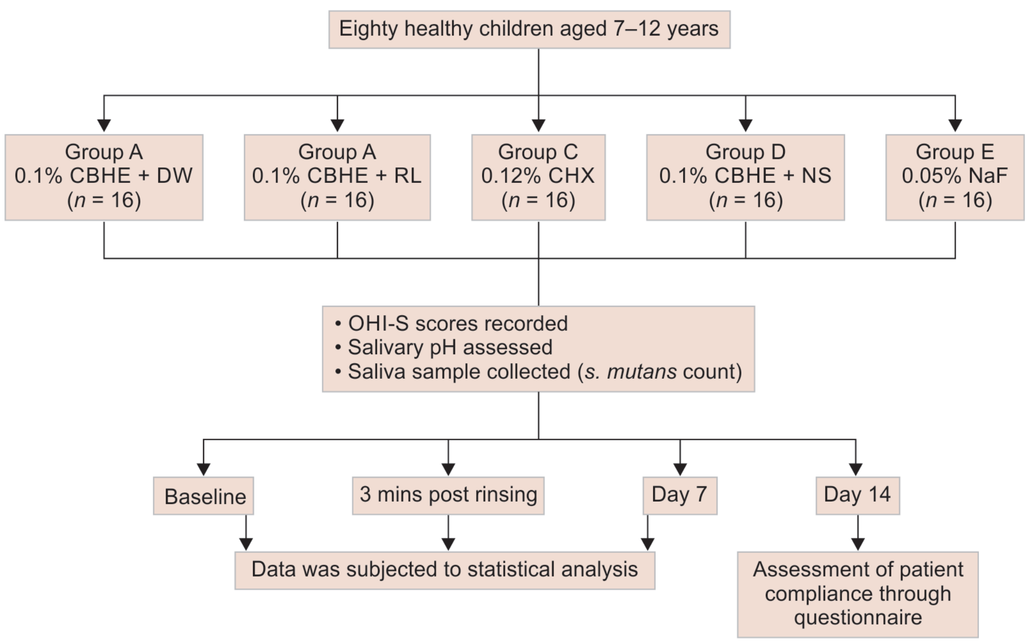

Fig. 1: CONSORT diagram

Fig. 2: Five different mouthwashes, namely group I—0.1% CBHE + DW, group II—0.1% CBHE + RL, group III—0.12% CHX, group IV—0.1% CBHE + NS, group V—0.05% NaF

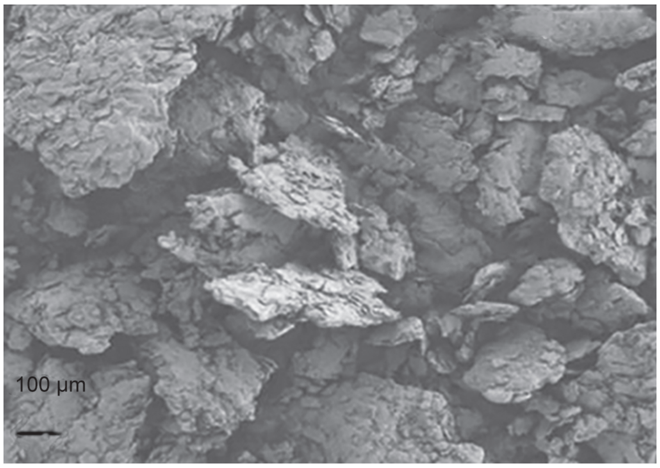

Fig. 3: CBH surface roughness at 100 μm magnification

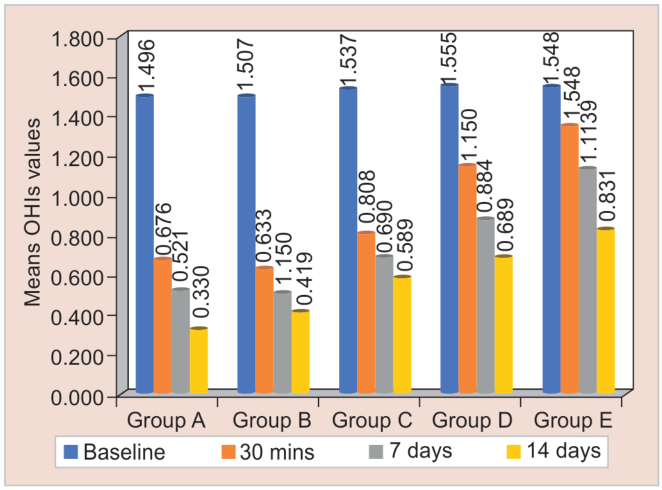

Fig. 4: Mean OHI-S scores between different time intervals in each group

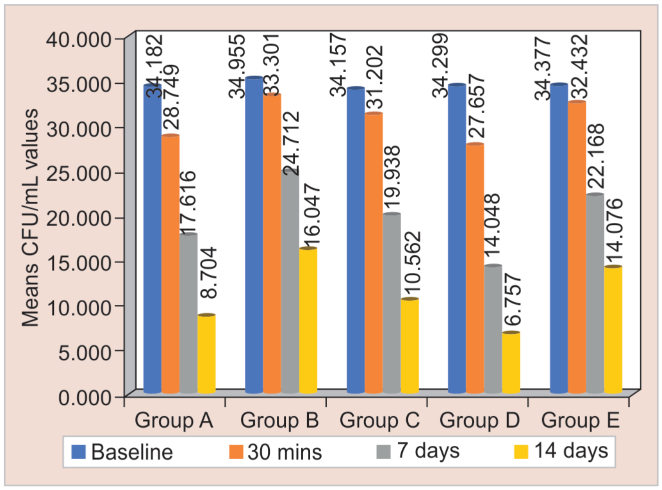

Fig. 5: Mean CFUs/mL between different time intervals in each group

determined; unstimulated saliva samples were collected on day 7 and day 14. At the end of the study, a validated questionnaire was given to the children for assessment of patient compliance and acceptance.

Saliva samples were inoculated onto the *mitis salivarius* agar plates and incubated for 48–72 hours at a temperature of 37°C. The colonies were counted and mean colony-forming units (CFUs) of different groups were determined. All values were tabulated and subjected to statistical analysis.

Statistical Analysis

Comprehensive descriptive analysis of all the demographic and knowledge, attitude, and practices variables were done using mean and standard deviation for quantitative variables, frequency, and proportions for categorical variables. Windows version 22.0 with Statistical Package for the Social Sciences was used to perform statistical analyses.

One-way Analysis of Variance (ANOVA) test, followed by Tukey's *post hoc* test was used to compare the mean OHIs, CFUs scores, and pH values between five groups at different time intervals. Repeated measures of the ANOVA test followed by Bonferroni's *post hoc* test were used to compare the mean OHIs, CFUs/mL, and pH values between different time intervals in each study group.

Chi-squared test was used to compare the responses to the study questionnaire between five groups. The level of significance (p-value) was set at $p < 0.05$.

Results

CBHE Particle Analysis

The particle size estimated for CBH was in the range of 0.69–0.78 μm and had a solubility of 88.6%. The CBH particle texture was observed at the magnification of 1000*X–100*X. The overall shape had an angularity and sphericity with a Feret angle of 84.32° (Fig. 3). The size of a round homogeneous molecule was particularly characterized by its width.

In the current study with GC-MS analysis, we found out that the amount of theobromine was 4.2–5.6 mg/gm% with a concentration of 0.2–2.0%/mL of the standard solution. However, the amount of catechin was 10.6–11.2 mg/gm% with a concentration of 0.4–1.0%/mL and that of epicatechin was 28.1–31.8 mg/gm% with a concentration of 0.2–1.0%/mL of the standard solution.

Anti-plaque Property (Fig. 4)

On comparing group I, that is, 0.1% CBHE with DW; group II, that is, 0.1% CBHE with RL; and group III, that is, 0.12% CHX collectively; with the 0.1% CBHE with NS (group IV) and 0.05% NaF (group V);

Table 1: Multiple comparisons of mean difference in pH values between different time intervals in each group using Bonferroni's *post hoc* test

Groups	*BL vs 30m*	*BL vs D7*	*BL vs D14*	*30m vs D7*	*30m vs D14*	*D7 vs D14*
Group I	<0.001*	0.03*	0.004*	1.00	1.00	1.00
Group II	0.03*	0.003*	0.17	1.00	1.00	0.98
Group III	<0.001*	0.003*	0.007*	1.00	1.00	1.00
Group IV	0.03*	0.04*	0.04*	1.00	1.00	1.00
Group V	0.02*	0.02*	0.01*	1.00	1.00	1.00

*, Statistically significant; 30m, post 30 minutes of rinsing; BL, baseline; D7, at day 7; D14, at day 14

groups I, II, and III showed a significant reduction in the mean OHIs scores after 30 minutes of intervention ($p < 0.001$). However, no significant difference was observed among the groups I, II, and III. Anti-plaque effectiveness was observed in all groups at day 7 with no statistically significant difference between 0.1% CBHE with DW, 0.1% CBHE with RL, and 0.12% CHX. However, 0.12% CHX and 0.05% NaF mouthwash groups showed significant differences. The mean scores of OHI-S recorded at the end of the study, that is, at day 14, were consistently lower in all the five groups, suggesting 0.1% CBHE with DW, followed by 0.1% CBHE with RL, and 0.12% CHX in comparable range of 0.330–0.589 to be more promising than CBHE with NS and 0.05% NaF groups ($p < 0.001$).

Antibacterial Property

The mean CFUs/mL in all five groups at 30 minutes postrinsing showed a significant difference from the BL value, with a maximum reduction observed in group IV (27.66×10^4), while the least was observed in group II (33.30×10^4). The mean colony count at day 7 revealed that 0.1% CBHE with NS showed a significant reduction to 14.048×10^4, followed by 0.1% CBHE with DW (17.616×10^4), and CHX (19.938×10^4). At the end of the study, it was significantly lowered, with the least reduction shown by 0.1% CBHE with NS, followed by 0.1% CBHE with DW, as shown in Figure 5.

Salivary pH and Patient Compliance

The mean difference in pH values in all the groups was statistically significant between BL and 30 minutes postrinse, BL and day 7, and BL and day 14, except group II, which showed no significant change between BL and day 14. The intergroup comparison of mean pH values between different time intervals within each group, there was no significant difference observed (Table 1).

Children were assessed for their likeness toward the provided mouthwash, for which 87.5% of group IV subjects liked the taste of mouthrinse, followed by group II (81.3%), group I = group V (75%), and group III (68.8%). A sweet, acceptable taste was reported by 81.3% of group II, 68.8% of group III, 56.3% of group IV, and 62.5% of group V, while 56.3% of group I mouthwash group reported with a bitter taste. Almost 75% of group I and 68.8% of group II subjects suggested the taste resemblance to chocolate compared to others ($p < 0.001$). A major proportion of subjects in all the groups did not experience any unpleasant sensations, such as vomiting, excessive salivation, etc. during the use of the mouthwash. The children were motivated to maintain their oral hygiene and wanted to continue using the mouthwash, as depicted by 100% of group IV, 62.5% of group V, 56.3% of group I, and 31.3% of group II.

Discussion

Cocoa bean husk (CBH) is a bioactive compound rich substance,[12] which along with its high fiber content, renders antibacterial property[5]. During and after the process of fermentation, several polyphenols diffuse out from the cotyledon into the husk imparting this property.

Polyphenols are the most interesting and studied compounds in CBH that are mainly responsible for their biofunctional properties. Earlier studies done in the field of food processing and agriculture industry highlighted that epicatechin is the most abundant and commonly reported flavan-3-ol contained in CBH, amounting for 0.21–34.97 mg/gm of CBH, followed by catechin (0.18–4.50 mg/gm) and their dimers, procyanidin B1 (0.55–0.83 mg/gm), and procyanidin B2 (0.23–1.38 mg/gm).[13] Thus, in the present study, before preparing the mouthwash, characterization of CBH particles followed by determination of polyphenols was conducted in order to understand its efficient application in dentistry. The amount of epicatechin determined was 28.1–31.8 mg/gm, which was in accordance with previous literature[13] and higher than the values obtained by Arlorio et al.[4] (2.75 mg/gm). Also, the amount of catechin determined in the present study was 10.6–11.2 mg/gm, which was higher than the values derived by Rojo-Poveda et al.[13] and Hernández-Hernández et al.,[5] who showed that hydrothermal treatment at 200°C for 5 minutes yielded 5.67 mg/gm of catechin. This difference in values is attributed to the genotype of the plant used, geographical variations, agronomic factors, the type of fermentation process, and the method of extraction employed.

The main methylxanthines found in CBH are theobromine (3,7-dimethylxanthine) and caffeine (1,3,7-dimethylxanthine). The amount of theobromine in CBH has been reported to be 5–7-fold higher than caffeine.[14] It has been shown to improve the microhardness of tooth enamel which could potentially increase resistance to tooth decay. Sadeghpour and Carey found that theobromine protected teeth from decay better than fluoride. The amount of theobromine in 1 ounce of dark chocolate bar has a better effect on tooth hardness than the 1.1% prescribed NaF treatment.[15] From previous literature where characterization was attempted for food processing, the amount of theobromine reported was 0.39–1.83 mg/100 gm of dried CBH and 0.04–0.42 mg/100 gm of dried CBH for caffeine,[13] which was much lesser than the amount of theobromine estimated in the present study, that is, 4.2–5.6 mg/gm by GC-MS analysis. This result was closely in accordance with an earlier study having values of 3.90 mg/gm for raw CBH.[5]

In the present study, CBH was grounded into powder and the size of the particle was estimated, which ranged between 0.69 and 0.78 µm and had a solubility of 88.6%. With an increase in particle size, the time taken for dissolution as well as optical density increased, indicating the linear effect of particle size on dissolution. Also, each particle under 100× magnification had an angularity and sphericity with a Feret angle of 84.32° which was the first of its kind to be determined.

A number of studies have demonstrated the anticaries activity of CBH extract, revealing that it reduces the development of almost all oral streptococci studied in humans by decreasing their acid production. In addition, the synthesis of insoluble glucans by the GTF of *S. mutans* and *S. sobrinus* is significantly inhibited by CBH extract.[10,16–19] In the study carried out by Osawa et al.,[18] the cariostatic substances present in CBH were isolated and characterized. It was found that the fraction with the highest anti-GTF activity was the one with the highest polyphenol content with the presence of epicatechin polymers having intermolecular bonds C-4 and C-8 (C-6).[20] Kopp and Bradbury[21] patented a method for the extraction of a polyphenol-enriched or a theobromine-enriched fraction from CBH by extraction with an acidified ethanol solution. In the present study, CBH extract was prepared using ethanol followed by lyophilization to achieve a powdered form that was used to prepare the mouthwashes.

After 30 minutes of the first rinse, 0.1% CBHE with DW and RL proved better at dislodging plaque from tooth surfaces in comparison to 0.12% CHX mouthwash. The reduction in *S. mutans* count observed in groups I, III, and IV after 30 minutes of rinsing were in accordance with Mustamin,[8] where he showed a reduction from 59.10 CFU/mL to 9.40 CFU/mL after 30 minutes in 0.1% CBHE group. However, group II showed the poorest efficacy in bacterial reduction due to the presence of sodium lactate which facilitates *S. mutans'* growth, thereby limiting its use as a vehicle.

Anti-plaque effectiveness was observed in all groups on day 7, with no statistically significant difference between CBHE with DW, RL, and 0.12% CHX. This finding was in accordance with Dukle et al.,[7] thus establishing 0.1% CBHE mouthwash as a safe alternative to standard CHX in children. However, 0.12% CHX and 0.05% NaF mouthwash groups showed significant differences at day 7, which was in accordance with Hambire et al.[22]

The mean colony count at day 7 revealed that 0.1% CBHE with NS showed a significant reduction to 14.048×10^4, followed by 0.1% CBHE with DW (17.616×10^4), and CHX (19.938×10^4). CBHE with RL and NaF group showed the least decrease in mean CFUs/mL since the fluoride group had lesser antibacterial efficacy than even CHX, which probably occurred due to the dominant antimicrobial role of CHX. And this result was in accordance with a study done by Dehghani et al.[23] NaF was compared for the first time with CBHE and with different vehicle media suggesting CBHE to be better than fluoride.

The mean scores of OHI-S recorded at the end of the study, that is, at day 14, were consistently lower in all the five groups suggesting CBHE with DW, followed by CBHE with RL, and 0.12% CHX in a comparable range of 0.330–0.589 to be more promising than CBHE with NS and 0.05% NaF groups ($p < 0.001$). Thus, we can clearly conclude that plain CBHE and CBHE with RL were better than other mouthwashes in significantly preventing plaque deposition, which was not in accordance with the study conducted by Srikanth et al.[6], Dua et al.,[24] and Shetty et al.,[25] who suggested CBHE as only a safe alternative to CHX. The probable cause for the inhibition of plaque deposition was the reduction of the hydrophobicity on the cell surface of *S. mutans* caused by polyphenols. Activity against *S. mutans* due to the fatty acids contained in CBH has also been proposed, mainly due to oleic and linoleic acids.[25]

The mean scores of CFUs at the end of the study was significantly lowered, with the least shown by 0.1% CBHE with NS, followed by 0.1% CBHE with DW suggesting better antimicrobial property in comparison to 0.12% CHX, which was not in accordance with the study conducted by Venkatesh Babu et al.,[10] where 0.2% CHX was used. However, a comparison of 0.12% CHX with 0.05% NaF group showed results similar to Dehghani et al.[23]

According to Mary et al.,[26] after using mouthwash, oral pH rapidly becomes alkaline, eliminating the risk of tooth erosion because children have thinner enamel. In the present study, the mean salivary pH values recorded on day 14 consistently increased in all five groups suggesting the overall effect of using mouthwash as an adjunct therapy for promoting a favorable oral environment. This finding was in accordance with Lim,[27] who concluded that the pH of saliva increases after using mouthwash.

Overall, the children developed a likeness to all three mouthwashes of 0.1% CBHE due to the addition of NS or RL. Only 56.3% of the subjects of the 0.1% CBHE group with DW complained of a bitter taste,[6,10] and the rest of the groups mentioned a sweet taste because saccharin sodium was added in the mouthwashes leading to excellent patient compliance. Previous literature showed that several modifications were made to mask the bitter taste of chocolate mouthwash by adding xylitol[7] and honey,[25] showing satisfactory patient acceptance, although the accountability toward its own antimicrobial and anticariogenic property remained a matter of concern that influenced the actual efficacy of CBHE. Therefore, preparing chocolate/CBHE mouthwash with NS or RL instead of DW and adding saccharin sodium helped improve taste and patient compliance, and further did not deteriorate the properties of the CBH.

No side effects were observed in all the groups indicating the preparations and concentrations of mouthwashes used in the current study are child-friendly and safe for usage. A very mild taste of chocolate was observed by the CBHE groups, which increased their compliance with its usage. All the children enjoyed the experience and willingly wanted to continue its further use, thereby promoting patient education and awareness of oral hygiene among them.

To summarize, among the three 0.1% CBHE combinations with vehicles, such as RL, NS, and DW, the addition of RL increased the anti-plaque efficacy, while using NS decreased this property in comparison to plain CBHE and CHX. Also, the addition of NS significantly reduced the growth of *S. mutans* in comparison to other vehicle media—group IV > group I > group II at all time intervals.

All the 0.1% CBHE combinations and 0.12% CHX were better than 0.05% NaF in reducing mean plaque scores at all time intervals. Plain CBHE and CBHE with NS showed a significant bacterial reduction in comparison to 0.05% NaF, which was better than CBHE with RL due to the presence of sodium lactate favoring bacterial growth. All three 0.1% CBHE combinations showed a significant difference in comparison to 0.12% CHX, suggesting it is a better alternative.

Conclusion

From this study, the following conclusions can be drawn:

- The 0.1% CBHE with NS as a vehicle showed maximum antibacterial properties among all the groups at all time intervals.
- The addition of RL to CBHE increased anti-plaque efficacy in comparison to plain CBHE, 0.1% CBHE with NS, and 0.12% CHX mouthwash.
- Plain CBHE, 0.1% CBHE with NS, and 0.12% CHX showed a significant reduction in *S. mutans* in comparison to 0.05% NaF which, however, was better than 0.1% CBHE with RL.

- All the 0.1% CBHE combinations and 0.12% CHX mouthwash were better anti-plaque agents than the 0.05% NaF group at all time intervals.
- There was a consistent increase in the salivary pH of the children in all the groups, suggesting the CBHE groups have a positive effect on increasing oral pH to combat dental caries.
- Improving the CBHE mouthwash preparation with NS or RL with the addition of saccharin sodium increased the likeness and acceptance of its usage by the children.

Clinical Significance

Cocoa bean husk extract (CBHE) mouthwash is a better anticariogenic, nonalcoholic mouthwash compared to CHX and NaF, which can be safely used in children as a routine oral rinse and also for those with gingivitis and high-risk of caries.

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