Stevia and Peppermint Herbal Formulation Based Mouthwash and Its Efficacy in Antimicrobial and Cytotoxic Activities

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Authors’ contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: Dental caries is a condition induced by the microbial fermentation of dietary carbohydrates in the biofilm that is connected to the teeth, which results in the disintegration of local chemical surfaces of the teeth. The predominant microbial pathogen in the aetiology of caries is Streptococcus mutans. Caries can be avoided by brushing and flossing regularly, as well as using antimicrobial mouthwash.

Aim: The aim of the study is to prepare the Stevia and Peppermint formulation mouthwash and evaluate the antimicrobial property and cytotoxic effect of the formulated mouthwash at varying concentrations.

Materials and Methods: A herbal mouthwash consisting of Stevia and Peppermint as the chief ingredients was prepared in the laboratory and its antimicrobial activity of various concentrations (25 µL, 50 µL, 100 µL) against S. mutans, S. aureus, E. faecalis, C. albicans were tested by the agar well diffusion method. The cytotoxic activity of the prepared herbal mouthwash was checked using lethality of the nauplii which hatched from brine shrimp eggs, which was inoculated for over

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24 hours. The results obtained were statistically analysed using IBM SPSS software and the results interpreted in graphs and tables.

**Results:** The antimicrobial activity of prepared mouthwash against different microorganisms at 25 µL was significantly lesser than the standard but there was a concentration dependent increase in the antimicrobial property. The cytotoxic effect of formulated mouthwash was found to be within limits at all concentrations. At low concentration the cytotoxic effect was found to be negligible and the cytotoxic effect also had a concentration dependent increase.

**Conclusion:** The formulated Stevia and Peppermint mouthwash was found to have effective antimicrobial properties and negligible cytotoxic effect. The antimicrobial property against different microorganisms at 25 µL is significantly less than the standard but the antimicrobial property increases as the concentration increases.

**Keywords:** Stevia; peppermint; anticariogenic; cytotoxic; dental caries.

1. **INTRODUCTION**

Dental caries is a disease which is characterized by localized destruction of the susceptible dental hard tissue by acidic by products from bacterial fermentation of dietary carbohydrate [1]. The significance of microorganisms in the etiology of dental caries has been highlighted in the ecological plaque hypothesis [2]. Although *Streptococcus mutans* and *Lactobacillus* have been identified as the main Cariogenic organisms, the key circle effectively describes the interaction of the causative factor in dental caries, namely host, diet, microbes and time [3]. A prolonged interplay of these factors result in the loss of tooth structure in the form of carious lesions [3,4]. Cariogenic microorganisms can be effectively controlled by antimicrobial therapy, these are by the use of agents in the form of mouthwash, sprays, dentifrices, gels, varnishes, chewing gums, lozenges, chemical or synthetic agents such as CHX , triclosan , xylitol etc [5,6].

CHX has high substantivity which is a main reason for its superior antimicrobial effect since it is a broad spectrum antimicrobial, it's routine use can alter the microbial equilibrium in the mouth. [7]. Therefore, it should be prescribed in appropriate doses only in selected high risk patients for a short time. In contrast to synthetic chemicals, natural products, such as herbal extracts have been found to be biocompatible with the tissues and the body [8,9,10]. Herbal medicines have been tested over time as a solution for all oral health problems [11]. Extracts of tulsi, neem, green tea have been tested for their Anti Cariogenicity in *in vivo* studies [12].

*Stevia rebaudiana* is a herbaceous perennial plant of the family Asteraceae, In India the cultivation is mostly in Gujarat. The leaf extract is used to sweeten foods. The major components are glycosides namely stevia side and rebaudioside - A. The extract of this plant has been used traditionally for the treatment of diabetes [13]. It has been investigated as an anti hypertensive, anti hyperglycaemic and an antioxidant. Previously there was no research article done based on herbal formulation with the ingredients of Peppermint and Stevia that can possess antimicrobial activity and cytotoxic effects [14]. The use of herbal formulations which are alcohol free, with fewer chemical components can be more efficient and biocompatible.

Stevia has many properties which can be used efficiently along with Peppermint to improve the oral health of the individual. Furthermore the increase in the incidence of new and re-emerging infectious disease and the development of resistance to the antibiotics in current use, make it urgent to discover new antibacterial compounds with novel mechanisms of action. The screening of plant extracts are of great interest to scientists in the search for new drugs for effective treatment of several diseases.

Our team has extensive knowledge and research experience that has translated into high quality publications [15–27,28–32,33,34]. The aim of the study was to evaluate the antimicrobial activity and cytotoxic effect of peppermint and stevia herbal formulation

2. **MATERIALS AND METHODS**

The study was performed in the Blue Lab of Saveetha Dental College and Hospitals. Ethical clearance required for the study was obtained from the institutional committee. The study was performed from January 2021- February 2021.
2.1 Preparation of Stevia and Peppermint Extract

The powdered stevia and peppermint were measured to 10 g and were emptied into a beaker. 100 mL distilled water was added into the beaker. The mixture was mixed well and was subjected to boiling at 90 degree Celsius until the aqueous mixture was well concentrated. The concentrated mixture was then subjected to filtration. The solution was boiled, cooled down and filtered to obtain the extract. The extract was concentrated to 10% and transferred to an Eppendorf tube.

2.2 Preparation of Stevia and Peppermint Mouthwash

The mouthwash was prepared using 0.3 g of sucrose added to 0.001 g of sodium benzoate and 0.01g of sodium Lauryl sulfate. This mixture was then dissolved in 10 mL of distilled water. To this solution 600 µL of plant extract and 50 µL of peppermint oil was added as flavouring agent and final preparation of the mouthwash was done. The mouthwash was mixed well and used for further analysis.

2.3 Antimicrobial Activity of Stevia and Peppermint

In the present study, to test antimicrobial activity of stevia and peppermint mouthwash, agar well diffusion method was used. Selective agar medium plates were marked and divided into four equal parts, labelled for specific organisms and mouthwash. A fresh bacterial culture having 10^8 CFU/ ml was spread on agar plates with a glass spreader. A well of 10 mm diameter was punched off at previously marked petri plates into agar medium with sterile cup borer and then it was filled with 25 µL, 50 µL, 100 µL of stevia and peppermint mouthwash. Plates were placed for 30 minutes in a refrigerator for diffusion of mouthwash and then incubated at 37 degree celsius for 24 hours depending upon the bacterial species, until appearances of inhibition zone. Zone of inhibition was measured as a property of antimicrobial activity. Antibiotics (Amoxyrite) was used as a reference drug. Amoxyrite was crushed into fine powder. About 0.2 g of amoxyrite powder was measured using a digital analytical balance and was added to 20 mL of distilled water respectively. The solution was mixed well using a vortex. This was considered as the standard drug for the antimicrobial activity.

2.4 Brine Shrimp Lethality Assay for Cytotoxic Activity of Prepared Mouthwash

2.4.1 Salt water preparation

2 g of iodine free salt was made and dissolved in 200 mL of distilled water

2.4.2 Brine shrimp

The eggs of brine shrimp were obtained commercially. A small water tank containing brine was taken and brine shrimp eggs were incubated for 48 hours for hatching. After 24 hours the larvae were used for the experiment.

2.4.3 Procedure for brine shrimp leth assay

6 well ELISA plates were taken and 10-12 mL of salt water was filled. To that 10 nauplii were slowly added to each well which contained the mouthwash in varying concentrations (control, 5 µL, 10 µL, 20 µL, 40 µL and 80 µL). The plates were incubated after 24 hours. This procedure was repeated 3 times to obtain triplicate values. After 24 hours, the ELISA plates were observed and noted for number of live nauplii present and calculated by using the following formula (no. of dead nauplii ÷number of dead nauplii x number of live nauplii x 100)

2.5 Statistically Analysis

Comparison of zones of inhibition of various microorganisms at different concentrations and the standard was done using one way ANOVA followed by Tukeypost hoc test. All the analysis was done by IBM SPSS software version 23 (IBM). One way anova followed by Tukey's post hoc test was used for overall comparison and pairwise comparison between the standard and different concentrations of the formulation. The statistical significance was set at 95% confidence limit and p<0.05 was considered as statistically significant.

3. RESULTS

This in vitro study analysed the antimicrobial and cytotoxic effect of the stevia and peppermint mouthwash. In this study, we tested the ethanolic extract of stevia and peppermint plants for their antimicrobial activity against human pathogenic bacteria such as *S. mutans*, *S. aureus*, *E. faecalis*, *C. albicans*. The evaluation of
The antimicrobial properties of formulated mouthwash showed that, at 25 µL and 50 µL the antimicrobial activity against *S. mutans* was found to be significantly less than that of standard. At 100 µL the result obtained was equal to the standard. At 25 µL the antimicrobial activity against *S. aureus* was equal to the standard. At 50 µL and 100 µL the antimicrobial activity against *S. aureus* was significantly greater than the standard. The antimicrobial activity against *E. faecalis* at all the tested concentrations were found to be significantly less than the standard. The antimicrobial activity against *C. albicans* at varying concentration was found to be equal to that of the standard. Thus the antimicrobial activity of different microorganisms at 25 µL is significantly less than the standard but the antimicrobial property increases as the concentration increases. The result obtained for antimicrobial activity of stevia and peppermint mouthwash from ANOVA test was found to be *p*= 0.006 found to be statistically not significant for *S. mutans, S. aureus, E. faecalis C. albicans*. The results showed that the cytotoxic effect of the stevia and peppermint formulated mouthwash was negligible at low concentration. There was a slight increase in the cytotoxic effect as the concentration increased but all within limits as 60% of the nauplii were alive even at the highest concentration. Cytotoxic activity of stevia and peppermint mouthwash from ANOVA test was found to be insignificant which is (*p*=0.117).

**Fig. 1.** Bar graph shows the Antimicrobial activity of stevia and peppermint mouthwash at varying concentrations along with the positive control (amoxicillin). The concentration was plotted on the x axis and the zone of inhibition was plotted on the y axis. The blue colour in the bar depicts the *S.mutans* and the green colour denotes the *S.aureus* and the brown colour denotes the *E. faecalis* and the purple colour represents the *C.albicans*. At 25 µL, the antimicrobial activity against *S. mutans* was found to be significantly less than that of standard (*p*<0.05), whereas at 50µL and 100 µL it was not significantly different (*p*>0.05). At 25 µL the antimicrobial activity against *S. aureus* was not significantly different from the standard (*p*>0.05) whereas at 50 µL and 100 µL it was significantly greater than the standard (*p*<0.05). The antimicrobial activity against *E. faecalis* at all the tested concentrations were found to be significantly less than the standard (*p*<0.05). The antimicrobial activity against *C. albicans* at varying concentration was not significantly different from the standard (*p*>0.05) (one way anova followed by post hoc analysis).
Fig. 2. Bar graph shows the cytotoxic activity of stevia and peppermint mouthwash at varying concentrations. The x axis represents the different concentration and the y axis represents the number of alive nauplii. The blue colour represents the initial count of the brine shrimp and the green colour represents the final count of the brine shrimp after 24 hours. The results showed that the cytotoxic effect of the stevia and peppermint formulated mouthwash was negligible at low concentration. There was a slight increase in the cytotoxic effect as the concentration increased but all within limits as 60% of the nauplii were alive even at the highest concentration. There was no significant difference in the cytotoxic effect between the control and the various concentrations tested (p>0.05) (one way anova followed by post hoc analysis).

Table 1. ANOVA test for antimicrobial activity

<table>
<thead>
<tr>
<th>ANOVA</th>
<th>Sum of squares</th>
<th>df</th>
<th>Mean square</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
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<td>Between Groups</td>
<td>190.000</td>
<td>3</td>
<td>63.333</td>
<td>9.268</td>
</tr>
<tr>
<td></td>
<td>Within Groups</td>
<td>54.667</td>
<td>8</td>
<td>6.833</td>
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</tr>
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<td></td>
<td>Total</td>
<td>244.667</td>
<td>11</td>
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<tr>
<td>S. aureus</td>
<td>Between Groups</td>
<td>159.000</td>
<td>3</td>
<td>53.000</td>
<td>53.000</td>
</tr>
<tr>
<td></td>
<td>Within Groups</td>
<td>8.000</td>
<td>8</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>167.000</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. faecalis</td>
<td>Between Groups</td>
<td>2552.250</td>
<td>3</td>
<td>850.750</td>
<td>850.750</td>
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<tr>
<td></td>
<td>Within Groups</td>
<td>8.000</td>
<td>8</td>
<td>1.000</td>
<td></td>
</tr>
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<td>Total</td>
<td>2560.250</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. albicans</td>
<td>Between Groups</td>
<td>.000</td>
<td>3</td>
<td>.000</td>
<td>.000</td>
</tr>
<tr>
<td></td>
<td>Within Groups</td>
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<td>Total</td>
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<td>11</td>
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</table>
Table 2. Post hoc test for antimicrobial activity

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Concentration (I)</th>
<th>Concentration (J)</th>
<th>Mean difference (I-J)</th>
<th>Std.Error</th>
<th>Sig.</th>
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</thead>
<tbody>
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<td>Zone of inhibition of S. mutans</td>
<td>25 µL</td>
<td>100 µL</td>
<td>-4.667</td>
<td>2.134</td>
<td>0.207</td>
</tr>
<tr>
<td></td>
<td>100 µL</td>
<td>50 µL</td>
<td>-7.000</td>
<td>2.134</td>
<td>0.045</td>
</tr>
<tr>
<td>Zone of inhibition of S. aureus</td>
<td>25 µL</td>
<td>100 µL</td>
<td>-11.000</td>
<td>2.134</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>100 µL</td>
<td>50 µL</td>
<td>-6.333</td>
<td>2.134</td>
<td>0.703</td>
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<tr>
<td>Zone of inhibition of E. faecalis</td>
<td>25 µL</td>
<td>100 µL</td>
<td>-4.000</td>
<td>2.134</td>
<td>0.310</td>
</tr>
<tr>
<td></td>
<td>100 µL</td>
<td>50 µL</td>
<td>-7.000</td>
<td>0.816</td>
<td>0.000</td>
</tr>
<tr>
<td>Zone of inhibition of C. albicans</td>
<td>25 µL</td>
<td>100 µL</td>
<td>-9.000</td>
<td>0.816</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Table 3. ANOVA test for cytotoxic effect

<table>
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<th></th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brine shrimp count initial</td>
<td>Between Groups</td>
<td>.000</td>
<td>5</td>
<td>.000</td>
<td>. .</td>
</tr>
<tr>
<td></td>
<td>Within Groups</td>
<td>.000</td>
<td>12</td>
<td>.000</td>
<td>. .</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>.000</td>
<td>17</td>
<td>. .</td>
<td>. .</td>
</tr>
<tr>
<td>Brine shrimp count post</td>
<td>Between Groups</td>
<td>39.778</td>
<td>5</td>
<td>7.956</td>
<td>2.238</td>
</tr>
<tr>
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<td>Within Groups</td>
<td>42.667</td>
<td>12</td>
<td>3.556</td>
<td>. .</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>82.444</td>
<td>17</td>
<td>. .</td>
<td>. .</td>
</tr>
</tbody>
</table>

4. DISCUSSION

The study aimed to assess whether there was any antimicrobial and cytotoxic effect for the prepared Stevia and peppermint herbal formulation and to test its efficacy with the known standards. Zone of inhibition and Brine shrimp lethality assay showed the efficacy of the prepared herbal mouthwash. The results of the study revealed that the stevia and peppermint formulation based mouthwash is having significantly higher antimicrobial activity than the antibiotic (amoxyrite) against S. aureus at all the tested concentrations whereas against E. faecalis the antimicrobial effect was significantly lower than the antibiotic (amoxyrite). Against S. mutans, higher concentrations of the mouthwash had the antimicrobial effect comparable to that of the antibiotic (amoxyrite). The antimicrobial effect against C. albicans was very less for all the tested concentrations of the mouthwash and the antibiotic (amoxyrite). There was no statistically significant difference between them. The cytotoxic effect of the stevia and peppermint formulated mouthwash was negligible at low concentration. There was a
Table 4. Post hoc for cytotoxic effect

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>(I) Concentration</th>
<th>(J) Concentration</th>
<th>Mean difference (I-J)</th>
<th>Std.Error</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brine shrimp count (post)</td>
<td>5 µL</td>
<td>10 µL</td>
<td>.667</td>
<td>1.540</td>
<td>.99</td>
</tr>
<tr>
<td></td>
<td>20 µL</td>
<td>40 µL</td>
<td>1.667</td>
<td>1.540</td>
<td>.87</td>
</tr>
<tr>
<td></td>
<td>80 µL</td>
<td>control</td>
<td>3.333</td>
<td>1.540</td>
<td>.32</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>0.000</td>
<td>3.667</td>
<td>1.540</td>
<td>.23</td>
</tr>
<tr>
<td></td>
<td>10 µL</td>
<td>20 µL</td>
<td>1.000</td>
<td>1.540</td>
<td>.98</td>
</tr>
<tr>
<td></td>
<td>40 µL</td>
<td>80 µL</td>
<td>2.667</td>
<td>1.540</td>
<td>.53</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>80 µL</td>
<td>3.000</td>
<td>1.540</td>
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<tr>
<td></td>
<td>control</td>
<td>20 µL</td>
<td>-667</td>
<td>1.540</td>
<td>.99</td>
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<tr>
<td></td>
<td>control</td>
<td>40 µL</td>
<td>1.667</td>
<td>1.540</td>
<td>.87</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>80 µL</td>
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<td>1.540</td>
<td>.78</td>
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<td></td>
<td>control</td>
<td>40 µL</td>
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<tr>
<td></td>
<td>control</td>
<td>80 µL</td>
<td>-3.333</td>
<td>1.540</td>
<td>.23</td>
</tr>
</tbody>
</table>

Slight increase in the cytotoxic effect as the concentration increased but all within limits as 60% of the nauplii were alive even at the highest concentration.
effect as the concentration increased but all within limits as 60% of the nauplii were alive even at the highest concentration. The study conducted by Ganesan et al. 2018, showed the cytotoxic activity against HepG2 cells increased with increase in the concentration. Our results are supported by various other studies.

The controversial aspect of stevia, according to previous studies, is that it has various adverse effects such as nausea, dizziness, headache, fatigue, bloating, and diarrhea. These are more common when stevia is consumed in a dose-dependent manner. Peppermint, on the other hand, is well-known for its ability to help with digestion, nausea, and dizziness, making it an ideal blend for concealing the effects of stevia. The findings of this study suggest that Stevia and peppermint herbal combination is a potent antimicrobial agent and has negligible cytotoxic effect. These results have to be validated with further cell culture studies and in vivo studies to recommend the same for clinical usage.

5. CONCLUSION

Within the limitations of this study and from the evidence obtained it can be conclude that stevia and peppermint mouthwash was found to have negligible cytotoxic effect and good antimicrobial activity and can therefore can be used for the application in the medical field.

NOTE

The study highlights the efficacy of "herbal medicine" which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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