PREPARATION OF STEVIA AND PEPPERMINT FORMULATION BASED MOUTHWASH AND ITS ANTI MICROBIAL ACTIVITY AND CYTOTOXIC EFFECT

Running Title : Preparation of stevia and peppermint formulation based mouthwash and its anti cariogenicity activity and cytotoxic effect

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

RESULT:

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

KEY WORDS :

Stevia, Peppermint, Anticariogenic, cytotoxic , dental caries

INTRODUCTION:

Dental caries is a disease which is characterised by localised destruction of the susceptible dental hard tissue by acidic byproducts 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 invivo 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

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.

1)Preparation of stevia and peppermint extract

The powdered stevia and peppermint were measured to 10 gm 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 Epindroff tube.

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.

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 25µL, 50µL,100µL 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 0.2g 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.

4) **Brine shrimp lethality assay for cytotoxic activity of prepared mouthwash :**

Salt water preparation:

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

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 .

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, 20µl, 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)

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.

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. mutants, S. Aureus, E. faecalis,C. Albicans. The evaluation of 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 ($p=0.117$)

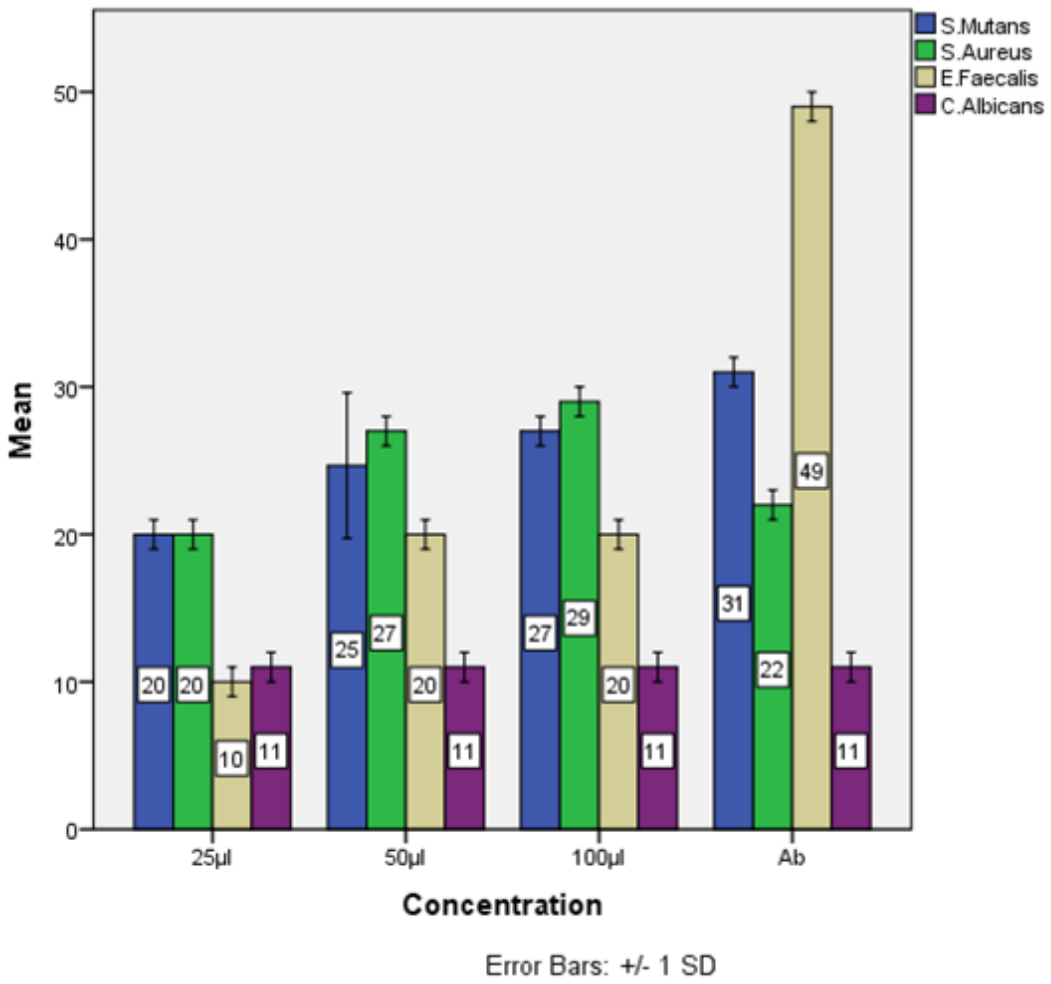

Figure 1 : Bar graph shows the Antimicrobial activity of stevia and peppermint mouthwash at varying concentrations along with the positive control(amoxicillin). The concentration was plotted on the x axis and the zone of inhibition was plotted on the y axis .The blue colour in the bar depicts the S.mutans 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)

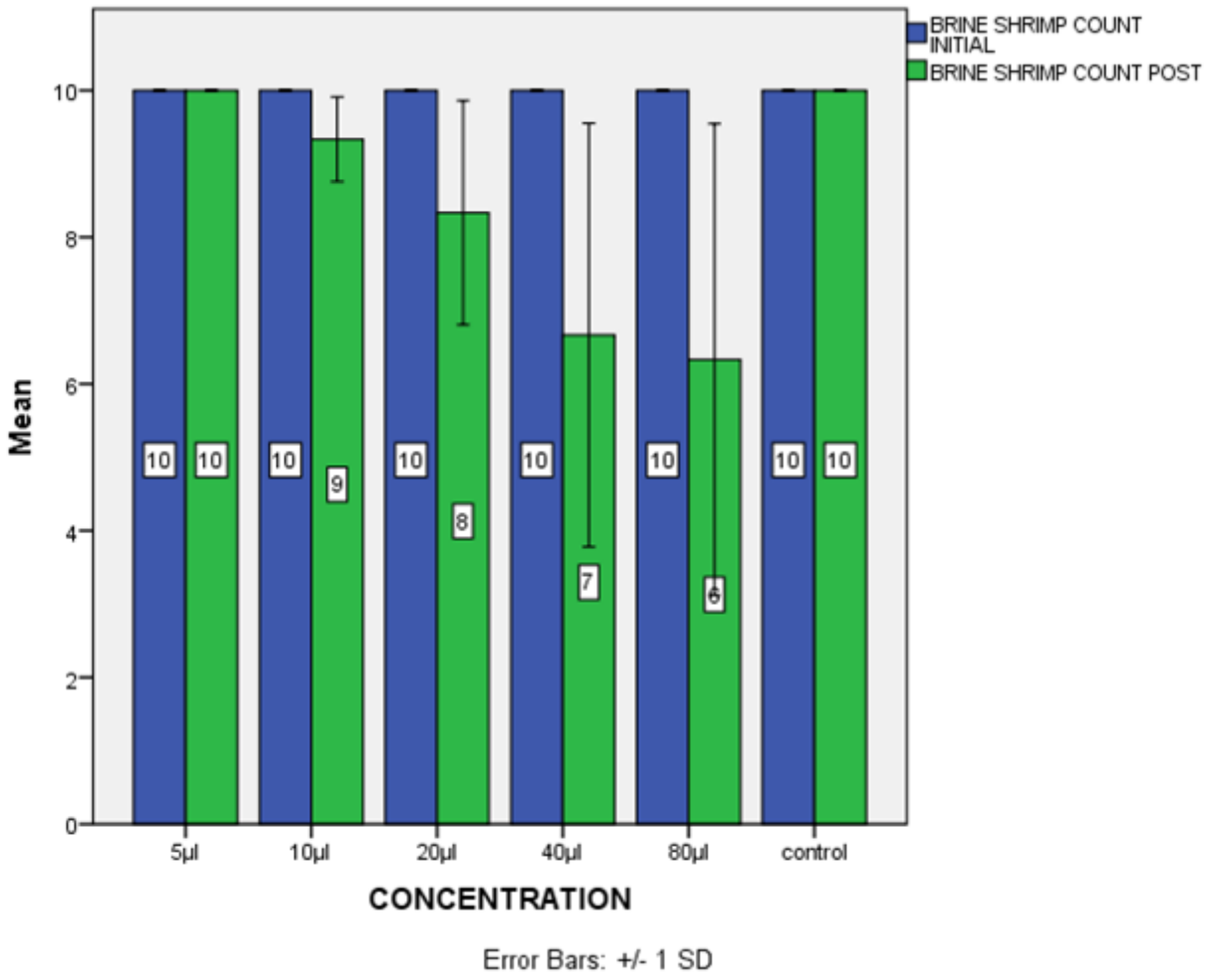

Figure2 : Bar graph shows the cytotoxic activity of stevia and peppermint mouthwash at varying concentrations.The x axis represents the different concentration and the y axis represents the number of alive nauplii. The blue colour represents the initial count of the brine shrimp and the green colour 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

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
S.Mutans	Between Groups	190.000	3	63.333	9.268	.006
	Within Groups	54.667	8	6.833		
	Total	244.667	11			
S.Aureus	Between Groups	159.000	3	53.000	53.000	.000
	Within Groups	8.000	8	1.000		
	Total	167.000	11			
E.Faecalis	Between Groups	2552.250	3	850.750	850.750	.000
	Within Groups	8.000	8	1.000		
	Total	2560.250	11			

C.Albicans	**Between Groups**	**.000**	**3**	**.000**	**.000**	**1.000**
	Within Groups	**8.000**	**8**	**1.000**		
	Total	**8.000**	**11**			

TABLE 2 : POST HOC TEST FOR ANTIMICROBIAL ACTIVITY

Dependent variable	(I) Concentration	(J) Concentration	Mean difference (I-J)	Std.Error	Sig.
	25	50µL	-4.667	2.134	0.207
Zone of inhibition of S. Mutans		100µL	-7.000	2.134	0.045
		Ab	-11.000	2.134	0.004
	50	100µL	-2.333	2.134	0.703
		Ab	-6.333	2.134	0.070
	100	Ab	-4.000	2.134	0.310
		50µL	-7.000	0.816	0.000
Zone of inhibition of S. Aureus	**25**	100µL	-9.000	0.816	0.000
		Ab	-2.000	0.816	0.144
	50	100	-2.000	0.816	0.144
		Ab	5.000	0.816	0.001

	100	Ab	7.000	0.816	0.000
		50µL	-10.000	0.816	0.000
Zone of inhibition of E. Faecalis	**25**	100µL	-10.000	0.816	0.000
		Ab	-39.000	0.816	0.000
	50	100	.000	0.816	1.000
		Ab	-29.000	0.816	0.000
	100	Ab	-29.000	0.816	0.000
		50µL	.000	0.816	1.000
Zone of inhibition of C.Albicans	**25**	100µL	.000	0.816	1.000
		Ab	.000	0.816	1.000
	50	100	.000	0.816	1.000
		Ab	.000	0.816	1.000
	100	Ab	.000	0.816	1.000

TABLE 3: ANOVA TEST FOR CYTOTOXIC EFFECT

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
BRINE SHRIMP COUNT INITIAL	Between Groups	.000	5	.000	.	.
	Within Groups	.000	12	.000		
	Total	.000	17			
BRINE SHRIMP COUNT POST	Between Groups	39.778	5	7.956	2.238	.117
	Within Groups	42.667	12	3.556		
	Total	82.444	17			

TABLE 4 : POST HOC FOR CYTOTOXIC EFFECT

Dependent variable	(I) Concentration	(J) Concentration	Mean difference (I-J)	Std.Error	Sig.
	5µL	10µL	.667	1.540	.99
		20µL	1.667	1.540	.87
		40µL	3.333	1.540	.32
		80µL	3.667	1.540	.23
		control	0.000	1.540	1.00
	10µL	20µL	1.000	1.540	.98
Brine shrimp count (post)		40µL	2.667	1.540	.53
		80µL	3.000	1.540	.42
		control	-667	1.540	.99
	20µL	40µL	1.667	1.540	.87
		80µL	2.000	1.540	.78
		control	-1.667	1.540	.87
	40µL	80µL	.333	1.540	1.00
		control	-3.333	1.540	.32
	80µL	control	-3.667	1.540	.23

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 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 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.

As per the caries management by Risk Assessment guidelines, the antimicrobial mouthwash is an important caries preventive therapy for prevention or control of dental caries in high risk individuals.(35) Apart from the use of well known mouthwashes such as CHX, there is a recent surge in the use of natural products as oral care therapies (36).The hypothesis generated in this study was based on the established antimicrobial properties of the glycosides from plant products on microorganisms. The leaves of stevia are a natural sweetener , ethanol and methanol extract of stevia and peppermint leaves have been found to be effective against gram negative and gram positive organisms.(35,37) In few in vitro studies mouthwash of stevia have been shown to be ineffective against S. Mutans . Despite such evidence, an aqueous extract of stevia leaves and peppermint formulation mouthwash was planned to evaluate the antimicrobial property.

The current study has used Amoxyrite as the standard to compare the antimicrobial activity of Stevia and peppermint since Amoxyrite is one of the most common choices of medication for treating bacterial infections. The zone of inhibition against S. mutans , S. aureus , E. faecalis and C. Albicans was tested using stevia and peppermint formulated mouthwash was found to be comparatively effective as that of the standard. Previous studies showed that Stevia rebaudiana

Bertoni leaves extracts as herbal mouthwashes are being tried and have been scientifically proven to be safe and effective to protection against dental caries because of its inhibitory effect or antimicrobial property against Cariogenic microorganisms. The antimicrobial efficacy of stevia and peppermint formulated mouthwash is attributed due to the leaves of stevia was found to be a natural sweetener , ethanol and methanol extract of stevia and peppermint leaves have been found to be effective against gram negative and gram positive organisms. Similarly the study conducted by Muanda et al. (2011) found that combination between essential oils plus extract of S. rebaudiana possess high antioxidants, anti-inflammatory and antimicrobial properties against S. aureus, B. subtilis, E. coli, P. aeruginosa, A. niger and C. albicans.(41,42) It has been reported that antimicrobial activity of the extracts of S. rebaudiana is due to the flavonoids, aromatic acids, terpenoids, phenolic acids and ester content.(43). The results obtained from various studies were in concordance with the current study.

The current study has used sodium chloride as the standard to compare the cytotoxic effect of Stevia and peppermint. Brine shrimp lethality assay was used to test the cytotoxic effect of the stevia and peppermint mouthwash. 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. 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 diarrhoea [47]. These are more common when stevia is consumed in a dose-dependent manner[48]. 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[49,50]. 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.

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.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. 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.

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