Original Articles

Cytotoxicity Evaluation of Herbal Mouthwashes Containing Ginseng Extract on Human Gingival Fibroblast-like Cells: An *In Vitro* Study

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Abstract

The study aimed to find an optimal concentrations of novel herbal mouthwashes which do not cause cytotoxicity to human gingival fibroblast-like cells (HGFs), according to ISO 10993-5, and to compare their cytotoxic effect to CUdent Stevia Fluoride™ mouthwash on HGFs. Three herbal extracts, ginseng (G), peppermint (P), and licorice (L), were dissolved in 25% v/v diluted CUdent Stevia Fluoride™ mouthwash to find the maximum dissolution. An optimal concentration of each herbal extract was evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The determined concentration was used to formulate the ginseng mouthwash solutions into two groups, ginseng-peppermint (GP) and ginseng-licorice (GL). Each group was tested once again by MTT assay to compare its cytotoxicity. One-sample *t*-test was used to analyze the determination of the cytotoxicity and one-way ANOVA to compare the cytotoxic effect between each group. The maximum dissolution of ginseng, peppermint, and licorice extract in CUdent Stevia Fluoride™ mouthwash was 5, 5, and 0.5 mg/mL respectively with no statistically significant cytotoxic effect (%viability ≥70%, $p>0.05$). When the novel mouthwashes were prepared, there was no statistically significant cytotoxic effect in any formulation (%viability ≥70%, $p>0.05$). Therefore, the cytotoxic effects on HGFs of every formulation were compared with CUdent Stevia Fluoride™ mouthwash, and they showed less cytotoxic effect ($p<0.05$). It can be concluded that the optimum concentration of ginseng, peppermint, and licorice extract is 0.5, 0.5, and 0.05 mg/mL. All formulas of ginseng mouthwashes in this study; pure ginseng solution (Gsol), ginseng-peppermint (GP), and ginseng-licorice (GL) showed less cytotoxic effect than the CUdent Stevia Fluoride™ mouthwash.

Keywords: Cytotoxicity, Ginseng mouthwash, Herbal mouthwash, Human gingival fibroblast-like cells

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Introduction

The periodontal disease is an inflammatory disease that affects periodontium or supporting structures of teeth.[1] Oral biofilm is considered the main ethiologic factor for the development of periodontitis. Thus, plaque control is an effective method in the elimination of biofim by mechanical and chemical means.[2,3] However, mechanical plaque control is restricted in some specific circumstances, for example, after oral surgery. In this situation, antimicrobial mouthwash could be used as chemical plaque control to delay biofilm accumulation together with tooth brushing and flossing.[4] Several antimicrobial chemical agents such as chlorhexidine (CHX) and cetylpyridinium chloride have been used. However, these chemical agents have unpleasant side effects such as taste stimulation and alteration, and staining on tooth surface and tongue, especially in a prolonged usage.[5] CHX is also cytotoxic, as reported for human gingival fibroblasts and osteosarcoma cells.[6] Currently, toxicity for oral cells is of potential concern. Therefore, nontoxic herbal mouthwashes using various herbs and plant extract have been introduced.

CU Dental Innovation Center has developed an herbal mouthwash containing stevia and peppermint extract. Peppermint oil has antimicrobial activity with minimum inhibitory concentration (MIC) ranging from 0.4% to 0.7% v/v depending on bacterial species.[7] Furthermore, it contains many components that have antioxidant activity, such as phenolic acids (eg. caffeic acid), flavones (eg.luteolin derivatives), and flavanones (eg. eriocitrin derivatives).[8] Stevia used as a sweetener has been extensively reviewed and approved as a food supplement in several countries.[9,10] Both peppermint and stevia have been reported to be associated with improving periodontal health.[11,12]

Licorice belongs to the genus *Glycyrrhiza*. It is one of the most popular natural agents in herbal mouthwashes. This extract has been used in treating gingivitis and periodontal diseases because of their antimicrobial and antioxidant effects.[13-16] In addition, licorice root extract has an antimicrobial effect on *P.gingivalis* with MIC of 62.5 µg/ml and MBC of 25 µg/ml and also affects the biofilm formation.[17]

Ginseng extract is now of interest for many researchers as it posseses plenty of medicinal values. It has been used in many forms such as orally or topically, especially in skin care products.[18] Ginseng root and leaf extract has antimicrobial, antioxidant effects and enhancees immune response.[19-21] Lee *et al.* [22] proposed that ginseng had anti-adhesive effects against certain periodontal pathogens. Ginseng used as an ingredient in many herbal mouthwashes and is proven its efficacy by comparing with commercially available mouthwashes and chlorhexidine.[23,24] Therefore, ginseng could be used as one of the essential ingredients in herbal mouthwash to improve periodontal status.

However, the International Standard for Oral hygiene products-Oral Rinses (ISO 16408) has identified that compatibility with oral tissues should be in accordance with ISO 10993-5 when assessing possible biological or toxicological hazards. Consequently, a compatibility test using the ISO 10993 method is required for developing herbal-containing mouthwash. The cytotoxicity test through the MTT assay is also a part of the process which indicates that if the cell viability is less than 70%, there is a possibility of cytotoxicity.[25] Generally, the oral mucosa comes in contact with the mouthwash when the oral cavity is flushed. However, in cases of injury, mothwash also comes in contact with the underlying gingival connective tissue.[26] Human gingival fibroblasts are commonly used to mimic connective tissue exposure to mouthwashes and to investigate cell-induced stress.[27,28] The purpose of this study was to find an optimal concentrations of novel herbal mouthwashes which do not cause cytotoxicity to human gingival fibroblast-like cells (HGFs), according to the ISO 10993-5, and to compare their cytotoxic effect to CUdent Stevia Fluoride™ mouthwash on HGFs.

Materials and Methods

1. Mouthwash preparation

The following herbal extracts were dissolved in 25% v/v CUdent Stevia Fluoride mouthwash (CUSF) (Fig 1) from 0.02% - 2% (w/v) to define the maximum dissolution: G = Ginseng root extract (Novanat, China); P = Peppermint powdered extract (Plantextrakt, Germany); and L = Licorice powdered extract (Plantextrakt, Germany).

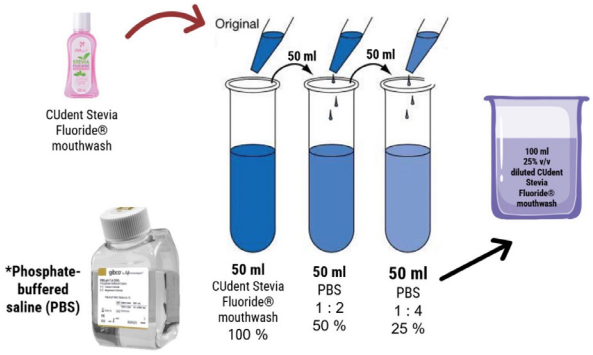

Figure 1 *Preparation of 25% v/v diluted CUdent Stevia fluoride mouthwash*

After the maximum dissolution of each herbal extract was defined (5 mg/mL of ginseng extract, 5 mg/mL of peppermint extract, and 0.5 mg/mL of licorice extract), the solution with less than or equal to the maximum dissolution was prepared for the cytotoxicity test through the MTT assay to define the optimum concentration (the maximum concentration which had no cytotoxicity effects to HGFs). The solution with an optimal concentration of G, P, and L was defined as G_{opt}, P_{opt}, and L_{opt} respectively.

The optimal concentrations were used to formulate 3 mouthwash groups; pure ginseng solution (G_{sol}), ginseng-peppermint (GP), and ginseng-licorice (GL). G_{sol} with various concentrations was prepared for the MTT assay in the previous step. GP was prepared in the following 5 formulations, which were made up of various proportions of G_{opt} and P_{opt}: $0.9G_{opt}+0.1P_{opt}$; $0.7G_{opt}+0.3P_{opt}$; $0.5G_{opt}+0.5P_{opt}$; $0.3G_{opt}+0.7P_{opt}$; and $0.1G_{opt}+0.9P_{opt}$. GL was prepared in the following 5 formulations, which were made up of various proportions of G_{opt} and L_{opt}: $0.9G_{opt}+0.1L_{opt}$; $0.7G_{opt}+0.3Lopt$; $0.5G_{opt}+0.5L_{opt}$; $0.3G_{opt}+0.7L_{opt}$; and $0.1G_{opt}+0.9L_{opt}$. Then GP and GL were prepared for the cytotoxicity test through the MTT assay.

2. Cell preparation

HGFs were obtained from healthy patients who underwent impacted tooth removal according to normal treatment plan at the Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Chulalongkorn University. The necrotic tissue was excluded. The samples were washed in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich Chemie, GmbH, Steinheim, Germany) supplemented with 1% v/v L-Glutamine (Gibco, Brazil), and 10% v/v Antibacterial-Antimycotic (Gibco, USA). The tissues were cut into 0.5×0.5 cm in size and placed in a new culture plate and then add to the culture media, DMEM supplement with 1% v/v L-Glutamine, 1% v/v Antibacterial-Antimycotic, and 10% v/v Fetal Bovine Serum (FBS) (Gibco, USA) at 37°C, 5% v/v CO_2, and 95% humidity.

After 5-7 days of incubation, the investigators examined the tissues under a microscope to ensure that fibroblast-like cells can be seen outside of the sample tissue and adhere to the surface of the plate. Culturing the cells until the 80-90% confluence was reached (changing of culture media every 3 days). The sample tissues were removed using forceps. Aspirating old culture media and washing with phosphate-buffered saline (PBS) solution were performed. 0.25% v/v Trypsin-EDTA 1 mL was added and incubated for one minute at 37°C, 5% v/v CO_2, and 95% humidity. The fetal bovine serum (FBS) (200 µl) was also added to inhibit the activity of trypsin enzyme. Cells were harvested and transferred into a 15 mL centrifuge tube. Centrifuging (Hermle LaborTechnik GmbH - Z 323 Universal High-Speed Centrifuge) at 3000 rpm for three minutes was performed. The supernatant was then aspirated, and the pellet was resuspended in culture media. Cell suspension was occasionally pipetted up and down to prevent the cells from settling and to ensure a uniform solution. Cells were counted and/or divided into a new culture dish to repeat the subculture procedure until the appropriate passage was reached.

The study protocol was approved by the Human Research Ethics Committee, Faculty of Dentistry of Chulalongkorn University (HREC-DCU 2020-046).

3. Cytotoxicity test

When the third or fourth passage was reached, cell cultures were taken from the culture flask. Cells were resuspended in culture media and the suspension was adjusted to a density of $1x10^5$ cell/mL before being dispensed into a 96-well tissue culture microtiter plate with a capacity of 100 µl each and was incubated for 24 hours at 37°C, 5%CO_2, and 95% humidity (Fig 2). A phase-contrast microscope was used to inspect each plate to ensure that cell growth was relatively uniform across the microtiter plate. Cell culture media was removed, and 100 µl of either the appropriate concentration of sample solution, negative control (culture media), positive control (non-ionic surfactant and emulsifier, TritonTMX-100), or blank (culture media in an empty well) was added according to ISO 10993-5. The sample solution was left on the cells for one minute before being withdrawn since the recommended treatment time when using mouthwash was for 30 seconds to 1 minute. The wells were gently rinsed with PBS and 100 µl culture media was added.

The culture media was removed after 24 hours of treatment and the plates were re-examined, and 50 µl of MTT solution (1 mg/ml) was added to each test well. The plates were incubated for another 2 hours at 37°C, 5%CO_2, and 95% humidity. The MTT solution (1 mg/ml) was discarded, and each well was filled with 100 µl of DMSO (Dimethyl sulfoxide for cell culture), (Sigma-Aldrich Chemie, GmbH, Steinheim, Germany). The experiment was done intriplicate. The plates were swayed and prepared for cell counting with a 570 nm filter to read the absorbance (OD570). The cell viability of HGFs of each herbal extract solution was analyzed quantitatively by an ELISA Reader (Bio-Tek Microplate Spectrophotometer Epoch II, Winooski, Vermont USA). All formulations of novel herbal mouthwashes were also tested and compared to CUSF (Fig 3).

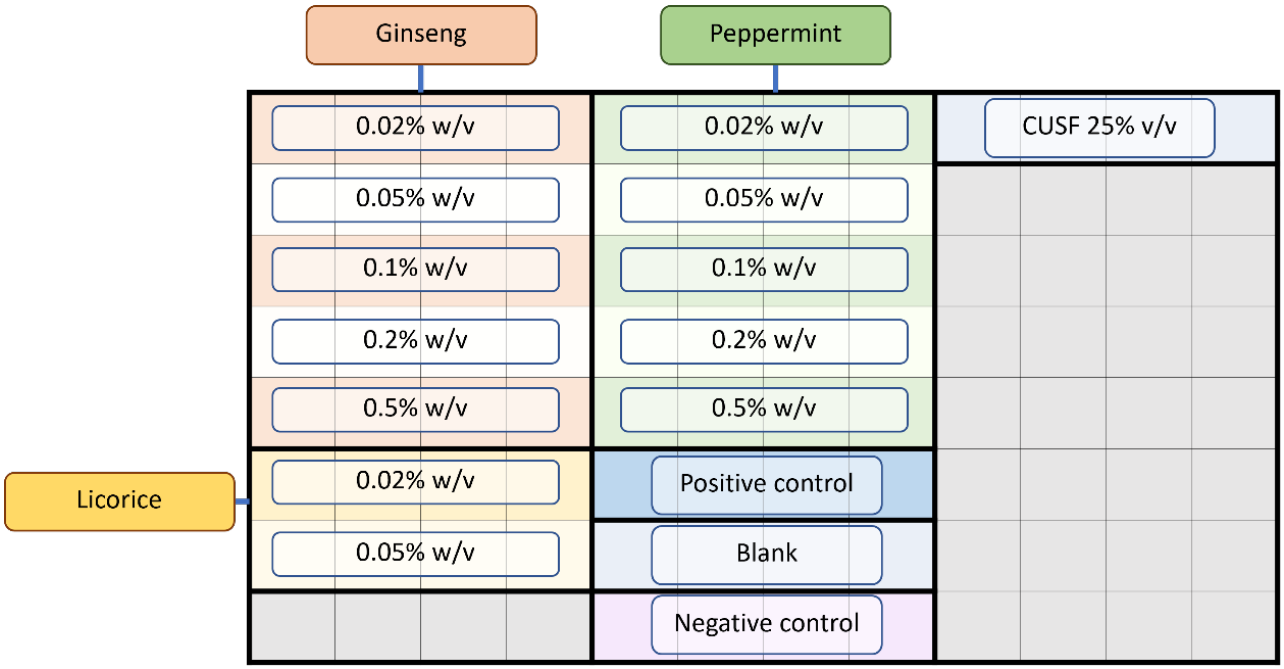

Figure 2 *The assay layout on a 96-well plate of three herbal extract solutions and in 25% v/v CUdent Stevia Fluoride mouthwash (CUSF)*

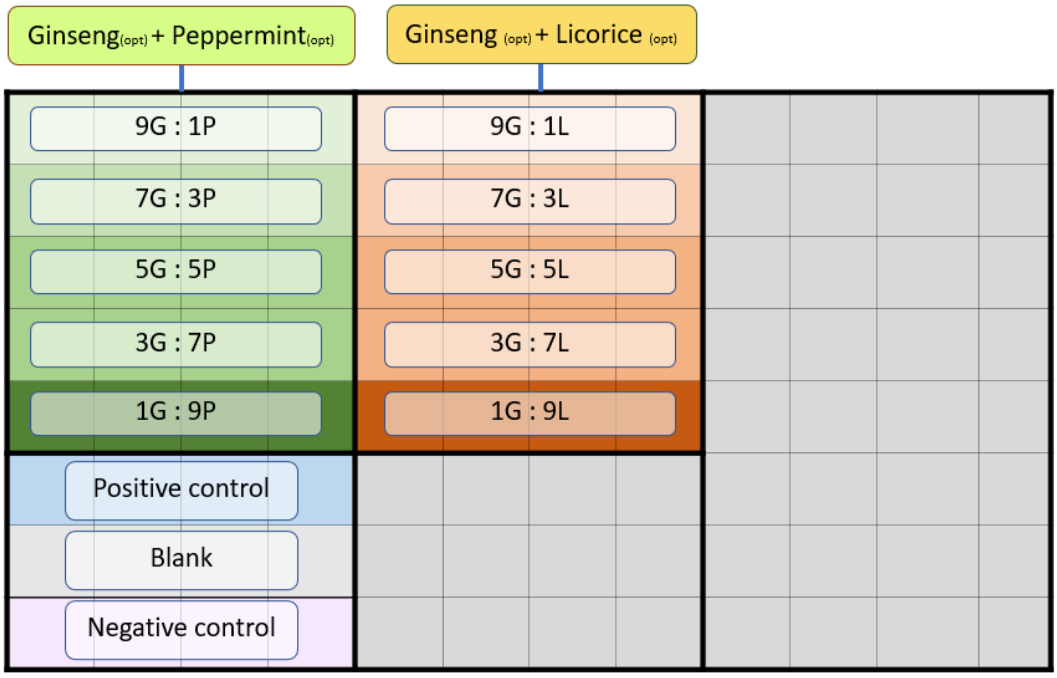

Figure 3 *The assay layout on a 96-well plate of the two formulations of novel mouthwash solutions (ginseng-peppermint and ginseng-licorice)*

The reduction of viability was determined based on the blank (%Viability) and calculated by the following equation.

$$\text{Viability \%} = \frac{OD_{570e}}{OD_{570b}} \times 100$$

where

OD_{570e} is the mean value of the measured optical density of the 100% extracts of the test sample.

OD_{570b} is the mean value of the measured optical density of the blanks.

Statistical analysis

Data analysis were performed utilizing the SPSS (IBM SPSS Statistic Version 26, SPSS Inc, Chicago, USA). The mean values of %viability, standard deviation, and 95% confidence interval were calculated for each group. One sample *t*-test was used for the assessment of statistical significance of cytotoxic potential of each group after the normality of data sample was tested by Shapiro-Wilk test. Cytotoxicity was defined as test samples with the %viability statistically less than 70%. One-way Analysis of variance followed by Dunnett's post hoc test was used to analyze the cytotoxic potential compared to CUdent Stevia Fluoride ™ mouthwash. A p-value < 0.05 was considered statistically significant.

Results

The optimal concentration of P, G, and L were found to be 0.5% w/v, 0.5% w/v, and 0.05% w/v with the mean value of %viability equals to 143.55, 96.30, and 108.70, respectively (Table 1). Moreover, no significant difference was identified between each herbal extract solution.

For the cytotoxic potential of novel mouthwashes formulated in this study, none of the mouthwashes had a significant cytotoxic effect on HGFs and had significantly higher %viability than the CUdent Stevia Fluoride™ mouthwash (Table 2).

Table 1 *Mean, standard deviation, and 95% confidence interval of %viability of each herbal extract solution in various concentrations*

Herbal extract solution	Mean (SD) of %Viability	95% Confidence interval		Shapiro-Wilk (*p*-value)
		Lower	Upper	
Peppermint				
0.02% w/v	139.54(26.14) [a]	118.22	160.85	0.371
0.05% w/v	138.95(27.14) [a]	116.82	161.08	0.073
0.1% w/v	140.71(25.58) [a]	119.85	161.56	0.550
0.2% w/v	138.14(24.74) [a]	117.97	158.31	0.448
0.5% w/v	143.55(24.67) [a]	123.44	163.66	0.412
Ginseng				
0.02% w/v	108.57(27.00) [a]	86.55	130.59	0.684
0.05% w/v	117.79(21.13) [a]	100.57	135.02	0.269
0.1% w/v	116.37(21.70) [a]	98.68	134.07	0.593
0.2% w/v	111.14(24.12) [a]	91.47	130.80	0.849
0.5% w/v	96.30(7.14) [a]	90.26	102.34	0.214
Licorice				
0.02% w/v	85.21(24.28) [a]	65.42	105.01	0.366
0.05% w/v	108.70(10.57) [a]	100.08	117.31	0.313
Control				
Positive control	0(0)	-	-	-
Negative control	100.00(5.06) [a]	98.00	102.00	0.733

[a]*The %Viability is not significantly lower than 70, which represents no cytotoxic potential. (ISO 10993-5)*

Table 2 *Mean, standard deviation, and 95% confidence interval of %viability of novel herbal mouthwashes formulated in this study, Gsol, and CUSF mouthwash*

Mouthwash	Mean (SD) of %Viability	95% Confidence interval		Shapiro-Wilk (*p*-value)
		Upper	Lower	
GP mouthwashes				
$0.9G_{opt}+0.1P_{opt}$	97.27(5.54)[a,b]	93.75	100.79	0.096
$0.7G_{opt}+0.3P_{opt}$	107.14(5.86)[a,b]	103.42	110.87	0.496
$0.5G_{opt}+0.5P_{opt}$	118.62(5.18)[a,b]	115.33	121.91	0.532
$0.3G_{opt}+0.7P_{opt}$	127.92(6.05)[a,b]	124.08	131.76	0.724
$0.1G_{opt}+0.9P_{opt}$	142.99(6.69)[a,b]	138.74	147.24	0.323
GL mouthwashes				
$0.9G_{opt}+0.1L_{opt}$	93.96(7.78)[a,b]	89.01	98.90	0.779
$0.7G_{opt}+0.3L_{opt}$	87.41(5.39)[a,b]	83.99	90.84	0.772
$0.5G_{opt}+0.5L_{opt}$	84.90(4.53)[a,b]	82.02	87.78	0.596
$0.3G_{opt}+0.7L_{opt}$	88.60(4.28)[a,b]	85.88	91.32	0.599
$0.1G_{opt}+0.9L_{opt}$	85.65(8.67)[a,b]	80.14	91.15	0.558
CUSF	77.39(8.59)[a]	71.93	82.85	0.628
Control				
Positive control	0(0)	-	-	-
Negative control	100.00(8.02)[a,b]	97.47	102.53	0.090

[a]*The %Viability is not significantly lower than 70, which represents no cytotoxic potential. (ISO 10993-5)*

[b]*Statistically significant difference from CUSF ($p<0.05$).*

Discussion

The purposes of the present *in vitro* study were to find an optimal concentrations of novel herbal mouthwashes which do not cause cytotoxicity to HGFs, according to the ISO 10993-5, and to compare their cytotoxic effect to CUdent Stevia Fluoride™ mouthwash on HGFs. The results showed that the three herbal mouthwashes including G_{sol}, GP, and GL had no cytotoxic effect more than the CUSF which is used as a solvent. These findings supported that addition of herbs containing ingredients increases antioxidant properties of mouthwash.[13,14] Reactive oxygen species (ROS) are by-products of normal cell activity that play a role in cellular signaling. Overproduction of ROS at high levels induces cellular damage and cell death under oxidative stress, which is an imbalance between free radicals and antioxidants. An antioxidant system, in which antioxidant enzymes remove ROS, is used to keep ROS at tolerable levels.[25] In our study, the chemical substances in CUSF which are similar to commercially available mouthwashes can cause oxidative stress because the components not only contain Stevia as an active compound, but also contain other substances such as fluoride which promotes cell stress, including endoplasmic reticulum stress and oxidative stress. As a result of the stress, ameloblasts that are responsible for the creation of dental enamel are malfunctioned leading to dental fluorosis.[26] Consequently, the addition of the herb extracts could eliminate ROS, prevent cellular stress, and decrease cytotoxicity. Moreover, the herbal extracts mixed in CUSF might have an active effect particularly increasing cell viability and decreasing cell toxicity of CUSF. Interestingly, our results demonstrated that peppermint extract may have some potential effect on cell proliferation based on GP results. There has been limited data available on the mechanism of peppermint effect on gingival fibroblast cell growth, however, Modarresi *et al* [27] stated that Mentha piperita essential oil might be utilized to accelerate wound healing in infected mice by lowering bacterial count, edema, and inflammation while enhancing fibroblast migration, collagen synthesis, and re-epithelization. Therefore, a mechanism of cell proliferation could not be described clearly because there are many factors affecting the process, especially inflammatory cells that play a role in the wound healing. The study implies that there is an increase in fibroblast cell proliferation, so it is interesting that it could be further investigated.

It should be noted that oral keratinocytes are the more appropriate cellular model for testing the cytotoxicity of mouthwash as they indeed are the cells that are in contact with mouthwash during usage. However, the present study aims to investigate the cytotoxicity according to the standard protocol described in ISO 10993-5 which indicates that fibroblast should be used as the cellular model. Further investigation regarding the cytotoxicity in oral keratinocytes is necessitated.

In the present study, the CUSF formulation was diluted with PBS to 25% v/v CUSF to simulate the oral cavity condition because PBS has properties like saliva such as buffer capacity, pH, coefficient of friction, and corrosion parameters.[28,29] Mystkowska *et al.*[28] reported that solvents including chlorhexidine (CHX), and neem extract solution were regarded as 100% solutions and were diluted into 0.1%, 1.0%, 10%, 25%, 50%, and 75% v/v for experimental purposes as in our pilot study. These findings supported that both CHX and neem extract demonstrated the mouthwash's cytotoxicity, with cells dying at 50% and 100% v/v concentrations, respectively. However, our study used 25% v/v CUSF owing to 50%v/v CUSF that was tested in our pilot study resulted in low cell viability (less than 70%viability) in some of replicates. Conversely, 25% v/v CUSF resulted in high cell viability (higher than 70%viability) in all replicates. The HGFs cells were also treated with mouthwash for 1 min, 5 mins, and 10 mins. However, treatment time for 30 seconds to 1 minute is the recommended time for mouthwash.

The maximum amount of herbs that can be dissolved without precipitation immediately after mixing in our pilot investigation was 0.2 g of herbs in 10 mL of solvent, the upper limit of the concentration range was

set at 2% w/v. Therefore, the precipitate emerged in solutions containing more than 0.05% w/v of licorice and more than 0.5% w/v of peppermint or ginseng. Furthermore, we chose 0.02% w/v as the lower limit of the concentration range because 0.02% w/v solution is made by combining 0.002 g of herbs with 10 mL of solvent, and 0.002 g is the smallest amount we can make with the least amount of mistake when preparing numerous times. Consequently, the concentration range was set at 0.02-2% w/v. The formulations aimed to see whether herbal mouthwashes had a cytotoxic effect on HGFs at different concentrations, but the therapeutic effect on the cells was not considered.

Interestingly, a mouthwash formula having only licorice has a cytotoxic impact that is inversely proportional to its concentration, which is the same as in a formula containing only peppermint in this study. It is most likely because the anti-cell-death components in the herbs are more concentrated, but the conclusion is unclear because we only had licorice in mouthwash at two concentrations in this study. However, the cytotoxic effect of mouthwashes containing both licorice and ginseng is increased when extra licorice is added. The cytotoxic effect may have been amplified by a chemical interaction between the components of each herb.

The mechanism of combined licorice and ginseng in the cells is unknown. The evidence of potential interaction between licorice and ginseng is limited. However, Popovich *et al.*[30] reported that the effects of ginseng and licorice extract combinations increase hepatocarcinoma cell viability and there were more cytotoxic than individual extract. On the other hand, our study showed that the combinations of extract were less cytotoxic because their actions were antagonistic rather than synergistic. The active compounds in ginseng and licorice extract may compete for the same cellular receptor, which could explain the antagonistic effect on cell viability. The discrepancy could be since the effect of combined licorice and ginseng on each cell were distinct, necessitating further investigation to validate their effects.[31]

There is a wide variety of herbal mouthwashes commonly used in the market. Besides active ingredients i.e. herbal extract, the other component may also differ among formulas. Sweetener is one of the key components to suit the user experience. The CUSF contains stevia as the sweetener while sodium saccharin is commonly used in general mouthwashes. In addition, CUSF contains fluoride which effectively prevents dental caries. However, the herbal mouthwash in general exhibits antimicrobial activity which also reduces the potential of caries formation as the cariogenic related bacteria is reduced. Other chemical agents are also introduced in the herbal mouthwash formula for other specific purposes i.e. adjuvant, solvent, or preservatives. Hence, the effect of a herbal mouthwash containing similar active herbal extract cannot be compared to those available in the market as the other components in the formula differ. According to the results of this study, although we found the proper concentrations for the novel mouthwash formulation, our study focused on cytotoxicity of novel herbal mouthwash, not the efficacy. The present study was in accordance with the ISO 10993-5.

The three selected herbs which are ginseng, peppermint, and licorice in the mouthwash did not show cytotoxicity to the tested cells, implying the safe application. These findings are also consistent with previous studies.[5,6,31,32] It may be concluded that the commercially available mouthwash which contains these active ingredients above the proper concentration could lead to cytotoxicity.

Conclusion

The optimal concentrations, which show no cytotoxicity according to ISO 10993-5 regarding the biological evaluation of medical devices, of ginseng, peppermint, and licorice extract in our novel herbal mouthwashes are 0.5, 0.5, and 0.05 mg/mL respectively. All three formulations of ginseng mouthwash which are G_{sol}, GP, and GL show less cytotoxic effect than CUSF. Further studies should evaluate therapeutic effect of

novel herbal mouthwashes and compare with commercially available mouthwashes and CHX for the treatment of periodontal diseases.

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