

## *In Vitro* Bioactivities of Alcohol-Free Benzydamine Oromucosal Solutions

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### Abstract:

**Objective:** This research aimed to evaluate the effects of chitosan and poloxamer 407 on *in vitro* cytotoxicity, anti-inflammatory, wound healing, and antimicrobial activities of 0.3% w/v benzydamine hydrochloride (HCl) oromucosal solutions.

**Material and Methods:** The effects of three alcohol-free benzydamine HCl oromucosal formulations; containing: 1) 0.5% w/v chitosan, 2) 15% w/v poloxamer 407, and 3) a combination of 0.5% w/v chitosan and 15% w/v poloxamer 407, on *in vitro* cytotoxicity and biological activities were evaluated and compared to a commercial benzydamine HCl mouth spray, containing alcohol and 0.12% chlorhexidine solution.

**Results:** All alcohol-free benzydamine HCl formulations and their vehicles were less cytotoxic to the macrophage RAW 264.7 cell line than the commercial spray and to the human gingival fibroblast cell line than chlorhexidine, respectively. The formulation containing 0.5% w/v chitosan exhibited the highest wound healing activity on the fibroblast cells among all tested products, and showed anti-inflammatory activity on macrophage cells, which is comparable to benzydamine HCl. Furthermore, from the time-kill assay, this formulation completely inhibited *Streptococcus mutans* within 1 minute, similar to 0.12% chlorhexidine solution, and reduced the cell number of *Candida albicans* more rapidly than the commercial spray.

**Conclusion:** The alcohol-free benzydamine HCl solution containing 0.5% w/v chitosan as a mucoadhesive polymer is a promising oral care candidate, which is safe to be used, and has wound healing, anti-inflammatory, and antimicrobial activities.

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**Keywords:** alcohol-free, benzydamine, bioactivity, chitosan, poloxamer 407, oromucosal solution

## Introduction

Oral mucositis is frequently observed in cancer patients undergoing chemotherapy, radiotherapy, or targeted therapy, causing cellular damage and ulceration<sup>1</sup>. Several approaches and agents for preventing and treating oral mucositis are available, with variable efficacy. Oral mucositis can be managed by using artificial saliva, rinsing the mouth with saline, sodium bicarbonate, or special mouthwashes containing topical anesthetics (2% viscous lidocaine), mucosal coating agents, opioid analgesics; anti-inflammatory agents (benzydamine), antimicrobial agents, and antiseptic agents. Patients must avoid alcohol or alcohol-containing oral care products; additionally, Chlorhexidine mouthwash or other topical antimicrobials are not recommended for prophylaxis or treating oral mucositis caused by chemotherapy; except when oral hygiene is poor. On the other hand, benzydamine; a non-steroidal anti-inflammatory drug, has been recommended for patients with head and neck cancer undergoing radiation without chemotherapy; due to its anti-inflammatory, pain relieving, antipyretic, and antimicrobial activities<sup>1-3</sup>.

Commercial benzydamine oromucosal solutions are available as mouthwashes and mouth sprays; however, there are some limitations due to a stinging sensation and short action. In addition, the pH of some commercial products is about 5, which promotes the erosion of teeth<sup>4</sup>. Moreover, formulations containing alcohol and strong flavoring agents can irritate and promote both dry mouth and inflammation, as the benzydamine is rapidly cleared from the mucosa by saliva flow. Benzydamine gel containing hydroxypropyl methyl cellulose has shown a statistically significant increase in the rate of oral mucosal repair in an animal model<sup>5</sup>. Therefore, alcohol-free benzydamine formulations with hydrating mucoadhesive polymers might be more appropriate to sustain the benzydamine in the oral cavity, as well as improving its pharmacological activities.

Chitosan is a cationic polysaccharide that possesses beneficial biological properties; such as biocompatibility, biodegradability, lack of allergenicity, non-toxicity, bioadhesion, hemostatic, wound healing, anti-inflammatory, antimicrobial, and moisturizing effect<sup>6</sup>. In addition, oral care products containing chitosan have been reported to prevent caries, control biofilm formation, and decrease pain and ulceration of stomatitis symptoms<sup>7-11</sup>. Chitosan has also been shown to possess anti-inflammatory activity by reducing the interleukin-1 $\beta$ -stimulated prostaglandin E2 protein levels in gingival fibroblasts<sup>12</sup>. The combination of chitosan and other polymers; such as alginate, poloxamer, and polyethylene glycol, has been widely used in drug delivery systems to improve their properties and efficacy<sup>6,13-15</sup>. Poloxamer 407, a non-ionic surfactant, is widely used in mucosal drug delivery systems due to its self-assembly into micelles, thermal reversibility, and biocompatibility. Although, poloxamer 407 lacks antibacterial properties, it has been shown to reduce the adhesion and biofilm formation of *Streptococcus mutans* (*S. mutans*) and *Candida* species as well as enhancing the rate of the wound healing process. An oral moisturizer containing 0.5% w/v chitosan and 15% w/v poloxamer 407 has been reported to demonstrate strong anti-adhesion and anti-biofilm properties against *Candida* species; however, it exhibited weaker anti-adhesion and anti-biofilm effects against *S. mutans* than the oral moisturizer containing only chitosan<sup>15</sup>.

Based on previous findings, this study was designed to evaluate the effects of alcohol-free benzydamine HCl oromucosal formulations containing chitosan and poloxamer 407 on *in vitro* cytotoxicity, anti-inflammatory, wound healing, and antimicrobial activities against *S. mutans* and *C. Albicans*, and compare them to a commercial 0.3% benzydamine HCl mouth spray and 0.12% chlorhexidine digluconate solution.

## Material and Methods

Benzydamine HCl, chitosan (average viscosity molecular weight of 414 kDa; with a 91% degree of deacetylation), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich, USA. Poloxamer 407 was obtained from Chanjao Longevity Company Limited, Thailand. Other excipients were pharmaceutical grade. The commercial benzydamine spray, composed of benzydamine HCl (3 mg/mL), ethanol, saccharin sodium, methyl hydroxybenzoate, glycerin, polyethylene glycol-40 hydrogenated castor oil, purified water, and a mint flavor, was used. Chlorhexidine digluconate was a gift from Songklanagarind Hospital. *S. mutans* DMST 18777 and *C. albicans* DMST 5815 were obtained from the culture collection of the Department of Medical Sciences, Ministry of Public Health, Thailand.

### Alcohol-free benzydamine HCl oromucosal formulations

Three different oromucosal solutions of 0.3% w/v benzydamine HCl were formulated, by using 0.5% w/v chitosan, 15% w/v poloxamer 407, or a combination of 0.5% w/v chitosan and 15% w/v poloxamer 407; namely C0.5P0, C0P15, and C0.5P15, respectively. The compositions of alcohol-free benzydamine HCl oromucosal formulations and their physicochemical properties are shown in Table 1. The oromucosal formulations were prepared by mixing an aqueous solution of chitosan, pH 6–6.5, and an aqueous solution composed of benzydamine HCl, poloxamer 407, along with other additives, pH 6–6.5, and then by adding purified water to the required volume. The pH was measured using a digital pH meter (Mettler, Toledo, Switzerland); the viscosity was determined using a viscometer (Model DV-III Ultra, Brookfield, USA) at 25°C. All formulations were physicochemical stable after storage at 30°C and 45°C, with a relative humidity of 75% for 3 months. Vehicles composed of all ingredients; except benzydamine HCl, and

0.3% benzydamine HCl in water, were prepared as control formulations.

### Anti-inflammatory activity studies and cytotoxicity in macrophage RAW 264.7 cell line

The inhibitory effect on nitric oxide (NO) production and cytotoxicity of samples using the macrophage RAW 264.7 cell line, a predominant producer of cytokines, were evaluated following the method of Tewtrakul et al. (2009)<sup>16</sup>. Briefly, the macrophage RAW 264.7 cells were seeded at a density of  $1 \times 10^5$  cells/well in a 96-well plate (100  $\mu$ L). After 1 hr of incubation at 37 °C in a humidified atmosphere containing 5% carbon dioxide (CO<sub>2</sub>), the medium in a 96-well plate was replaced with a fresh medium containing of 0.2  $\mu$ g/mL of lipopolysaccharide (100  $\mu$ L), along with 100  $\mu$ L of samples in the ranges of concentrations at 1.7–13.6 mg/mL; equivalent to benzydamine HCl 5–40  $\mu$ g/mL. Then, the plates were incubated for 48 hr. The NO production was evaluated by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent at a 1:1 ratio. The density of NO production was measured at 550 nm using a microplate reader. The anti-inflammatory activity of the developed formulations were compared to benzydamine HCl and a commercial benzydamine product. Caffeic acid phenethylester (nuclear factor kappa B inhibitor), at the range of concentration 0.5–5  $\mu$ g/mL and indomethacin (cyclooxygenase and inducible nitric oxide synthase inhibitor) at the range of concentration 1–40  $\mu$ g/mL, were used as standard positive controls. Cytotoxicity was determined using the MTT colorimetric method. The sample was considered cytotoxic if the density of the sample-treated group was less than 80% of the control group.

### Wound healing by scratch test assay in a human gingival fibroblast (HGF) cell line

The scratch test assay was used to assess the wound healing activity of the sample, following the method

of Chaitrakoonthong et al. (2020)<sup>17</sup>. Briefly, HGF cells were seeded at a density of  $1 \times 10^5$  cells/well in a 12-well plate and incubated for 24 hr at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub> incubator to allow cell adhesion. A straight-line scratch was created in the native cell monolayer using a P1000 pipette tip. A fresh medium, or sample at the concentration of 0.34 mg/mL and 1.7 mg/mL, equivalent to benzylamine HCl 1 µg/mL and 5 µg/mL; respectively, was added to each well and incubated at 37 °C. At 24, 48, and 72 hr, the wound area was observed and recorded using a phase-contrast inverted microscope. The remaining wound areas were analyzed using ImageJ software.

#### Antimicrobial and antibiofilm activities

Antimicrobial and antibiofilm activities against *S. mutans* DMST 18777 and *C. albicans* DMST 5815 of the alcohol-free benzylamine HCl formulations were determined in comparison with a commercial benzylamine spray and 0.12% w/v chlorhexidine solution.

The antimicrobial activity was evaluated using a colorimetric broth microdilution method, as described by Sungkharak et al. (2016).<sup>18</sup> The final concentrations of 0.3% benzylamine samples were in the range of 4.9–2500 µg/mL, equivalent to benzylamine HCl of 1.5–750 µg/mL, and the final concentrations of vehicles and chitosan solution were in the range of 9.8–5000 µg/mL. The results were expressed as the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of the samples. The lowest concentration showing a blue color was considered as the MIC value based on the lack of reduction of the blue resazurin to the pink resorufin by the microbial dehydrogenase enzyme. The aliquots of broth from the wells containing no growth were inoculated by the streak plate method. The complete absence of growth was considered to represent the MBC or MFC.

The antibiofilm activities were determined using a microplate-based assay, as described by Perumal and

Mahmud (2013)<sup>19</sup>. The results were expressed as minimum biofilm inhibition concentration (MBIC), which is defined as the lowest concentration of an antimicrobial agent required to inhibit the formation of biofilms, The minimum biofilm eradication concentration (MBEC) can be defined as the lowest concentration of an antimicrobial agent required to eradicate biofilm. In addition, a time-kill assay was performed according to the method of Nittayananta et al. (2018)<sup>20</sup>. The microbial suspensions were collected at different time points (1, 5, 15, 30, 60, 120, and 180 min) after exposure to the samples, and the reduction of microorganisms was evaluated using the standard plate count method.

#### Data analysis

All experiments were conducted in triplicate. The data are shown as the mean and standard deviation (S.D.). One-way analysis of variance (ANOVA) single factor was used to analyze the data, with the level of significance at a p-value less than 0.05.

## Results

#### Anti-inflammatory activity and cytotoxicity in macrophage RAW 264.7 cell line

*In vitro* cytotoxicity testing of the formulations was conducted by measuring the cellular mitochondrial activity as a function of concentration. The commercial spray and the alcohol-free benzylamine HCl solutions exhibited no or negligible cytotoxicity (% relative cell viability greater than 80%) to the macrophage RAW 264.7 cell line at concentrations of less than 3.4 and 6.8 mg/mL, equivalent to benzylamine HCl of 10 and 20 µg/mL, respectively. However, the vehicles of the formulations and the positive controls exhibited no cytotoxicity at the highest concentration tested. The IC<sub>50</sub> value of C0.5P0 was comparable to that of benzylamine HCl and higher than those of commercial spray, C0P15 and C0.5P15; as shown in Table 2.

The anti-inflammatory activity of the oromucosal solutions was tested by measuring their inhibitory effect on NO production assay in the macrophage RAW 264.7 cell line. Caffeic acid phenethyl ester possessed the highest inhibitory effect on NO production, with an  $IC_{50}$  value of  $0.91 \pm 0.01 \mu\text{g/mL}$ , following that of the commercial spray; as shown in Table 2. The  $IC_{50}$  value of indomethacin was  $14.88 \pm 0.63 \mu\text{g/mL}$ , which was comparable to those of benzylamine HCl and the oromucosal solutions containing mucoadhesive polymers. However, the vehicles of the formulations had no anti-inflammatory activity.

#### Wound healing by scratch test assay in HGF cell line

Formulation C0.5P0, equivalent to benzylamine HCl  $1 \mu\text{g/mL}$ , significantly accelerated the wound healing, as demonstrated by the proliferation and migration of HGF cells as well as complete wound closure after 72 hr of the scratch wound creator; as shown in Figure 1. Notably, HGF cells treated with C0.5P0 and its vehicle for 48 hr exhibited exceptionally high wound healing activity, with 95% and 100% closure, respectively. The alcohol-free benzylamine formulations showed a significant difference in cell migration to close the scratch wound compared to the control,

commercial spray, and chlorhexidine, while there were no differences between the formulations with mucoadhesive polymers and the benzylamine HCl in water. The wound healing activity of the solutions at a concentration equivalent to drug  $5 \mu\text{g/mL}$  was comparable to that of  $1 \mu\text{g/mL}$ . However, chlorhexidine ( $5 \mu\text{g/mL}$ ) was cytotoxic to HGF cells, and therefore no data of the percentage of wound healing is presented.

#### Antimicrobial and antibiofilm activities

The capability of C05P0 formulation to inhibit, kill, inhibit biofilm formation, and eradicate biofilm of *S. mutans* and *C. albicans* was in the range of concentration of benzylamine HCl and the commercial spray. However, the activities of benzylamine in various formulations were significantly lower than those of chlorhexidine; as shown in Table 3. Chitosan exhibited antimicrobial and antibiofilm activities against *S. mutans* and *C. albicans* at higher concentrations than benzylamine, while the vehicle had no such activities. There was no additive effect between benzylamine HCl and chitosan. From the time-kill assay, the C05P0 solution significantly decreased oral pathogens more rapidly than the commercial spray and C0.5P15; as shown in Figure 2.

**Table 1** Composition and physicochemical properties of alcohol-free benzylamine HCl oromucosal formulations

Composition (%)	C0.5P0	C0P15	C0.5P15
Benzylamine HCl	0.3	0.3	0.3
Chitosan	0.5	0	0.5
Poloxamer 407	0	15	15
Sodium saccharin	0.05	0.05	0.05
Raspberry flavor	0.2	0.2	0.2
Methylparaben	0.1	0.1	0.1
Propylparaben	0.02	0.02	0.02
Propylene glycol	0.88	0.88	0.88
Lactic acid/ 1 N NaOH qs to pH 6–6.5	qs	qs	qs
Purified water qs to	100	100	100
Physicochemical properties			
Appearance	Clear solution	Clear solution	Clear solution
pH* (25 °C)	$6.21 \pm 0.01$	$6.41 \pm 0.00$	$6.51 \pm 0.00$
Viscosity* (25 °C)	$12.80 \pm 0.35$	$123.37 \pm 1.93$	$281.54 \pm 1.83$

\*mean  $\pm$  S.D. (n=3), HCl=hydrochloride

**Table 2** The anti-inflammatory activity and relative cell viability of benzydamine HCl oromucosal solutions tested by inhibitory effect on nitric oxide (NO) production assay in macrophage RAW 264.7 cell line as a measurement of anti-inflammatory activity

Sample	% NO inhibition: IC <sub>50</sub> (µg/mL)*	% Relative cell viability: IC <sub>50</sub> (µg/mL)*
Indomethacin	14.88±0.63 <sup>b, c</sup>	NA
Caffeic acid phenethylester	0.91±0.01 <sup>a, c, d</sup>	NA
Commercial spray <sup>+</sup>	5.11±1.40 <sup>a, b, d</sup>	23.37±3.66 <sup>d</sup>
Benzydamine HCl	11.41±3.99 <sup>b, c</sup>	42.58±7.41 <sup>c</sup>
C0.5P0 <sup>+</sup>	9.19±1.27 <sup>a, b, c</sup>	41.60±0.59 <sup>c</sup>
C0P15 <sup>+</sup>	12.24±1.96 <sup>b, c</sup>	25.62±0.95 <sup>d</sup>
C0.5P15 <sup>+</sup>	12.67±1.97 <sup>b, c</sup>	28.61±1.55 <sup>d</sup>

\*mean±S.D., n=3, NA=not available

% NO inhibition=(A-B)/(A-C)×100, where; A=the absorbance of lipopolysaccharide supernatant without a sample, B=the absorbance of lipopolysaccharide supernatant with sample, C=the absorbance of supernatant without lipopolysaccharide and sample, IC<sub>50</sub>=the concentration that can inhibit NO cell secretion for 50%, <sup>+</sup>equivalent to benzydamine HCl

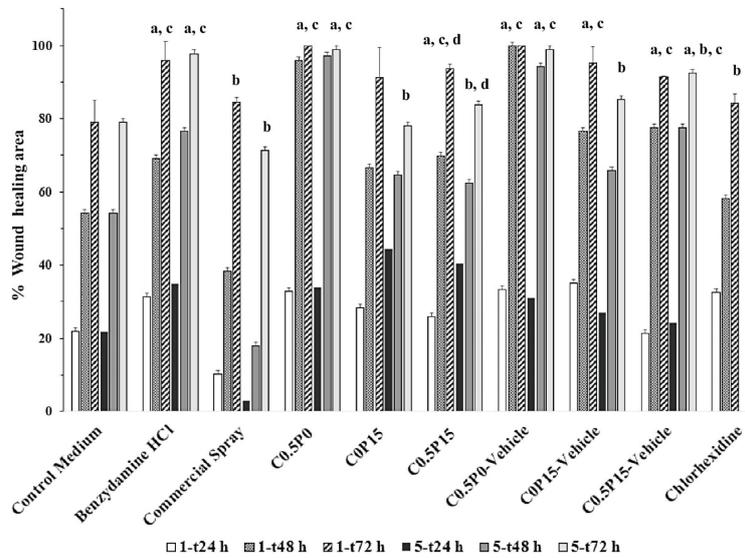
<sup>a, b, c, d</sup>=p-value<0.05 when compared with indomethacin, caffeic acid phenethylester, commercial spray, and benzydamine HCl, respectively  
HCl=hydrochloride

**Table 3** The antimicrobial and antibiofilm activities of oromucosal solutions against *S. mutans* and *C. albicans*

Samples (µg/mL)	<i>S. mutans</i>				<i>C. albicans</i>			
	MIC	MBC	MBIC	MBEC	MIC	MFC	MBIC	MBEC
Chlorhexidine digluconate	0.98	1.95	1.95	7.81	15.63	31.25	15.63	62.50
Benzydamine HCl	188	188	188	375	375	375	188	750
Commercial Spray <sup>+</sup>	375	750	375	375	375	750	94	>750
C0.5P0 <sup>+</sup>	188	>750	94	375	375	375	375	750
C0.5P0-Vehicle	5000	NI	-	-	NI	-	-	-
Chitosan	1250	5000	1250	2500	>5000	-	>5000	>5000

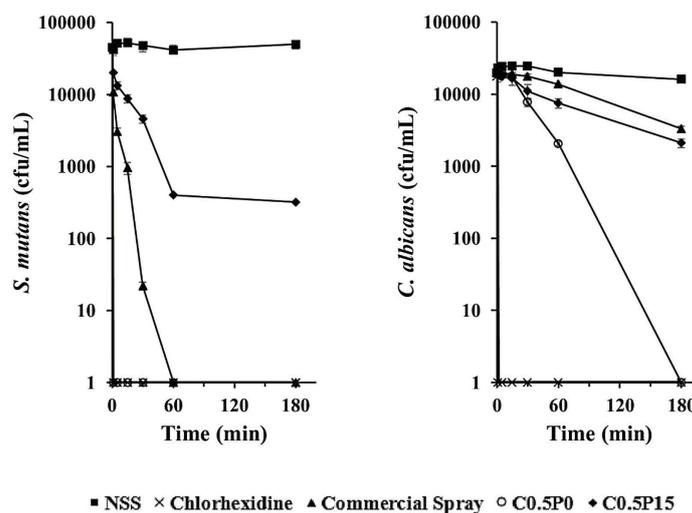
Data is expressed as mode (n=4), NI=Not inhibit, -=Not determined, <sup>+</sup>equivalent to benzydamine HCl

*S. mutans*=*Streptococcus mutans*, *C. albicans*=*Candida albicans*, MIC=minimum inhibitory concentration, MBC=minimum bactericidal concentration, MBIC=minimum biofilm inhibitory concentration, MBEC=minimum biofilm eradication concentration, MFC= minimum fungicidal concentration, HCl=hydrochloride



mean±S.D., n=3, % Wound healing=[(Wound area of t0–Wound area of tn)/(Wound area of t0)]x100 Where t0=Initial time of scratch test assay, tn=time of detection at 24, 48, or 72 hr.a, b, c, d=p-value<0.05 when compared with control medium, benzylamine HCl, commercial spray, and vehicle, respectively

**Figure 1** The wound healing activity of alcohol-free benzylamine HCl formulations and controls at the concentrations of 0.34 mg/mL and 1.70 mg/mL equivalent to benzylamine HCl and chlorhexidine 1 µg/mL and 5 µg/mL in human gingival fibroblast cell line



(NSS=normal saline solution, chlorhexidine=0.12% w/v chlorhexidine digluconate solution, and others=0.3% w/v benzylamine HCl oromucosal solutions) (mean±S.D., n=3)

**Figure 2** The reduction in cell number of *S. mutans* and *C. albicans* after exposure to oromucosal solutions for 180 min

## Discussion

The prevention and management of oral mucositis are critical aspects of care for patients undergoing anticancer treatment. The severity of symptoms must be carefully considered to determine appropriate interventions. One strategy is modifying the patient's diet to avoid rough and sharp-edged foods; such as potato chips, as well as oral care products containing alcohol and strong flavoring agents, which may cause incidental trauma or burning. In this study, alcohol-free benzylamine solutions containing hydrating mucoadhesive polymers and mild flavoring agents were developed to sustain the drug on the oral mucosa and improve safety, wound healing, and antimicrobial activities.

Two mucoadhesive polymers, chitosan and poloxamer 407, were used to enhance the bioactivity of benzylamine in the treatment of oral mucositis. Mouthwash containing 0.5% w/v chitosan has been reported to be more effective than chlorhexidine in relieving oral mucositis symptoms<sup>10</sup>. Poloxamer 407 was used at 15% in the solution due to its thermo-responsive property.

The safety of oromucosal solutions was evaluated by their cytotoxicity to macrophage RAW 264.7 cells. The results indicated that all alcohol-free formulations showed cytotoxicity at a higher concentration compared to the commercial spray. Moreover, the vehicle of the formulations was found to be safer than benzylamine formulations. These findings suggest that the presence of alcohol and benzylamine HCl may increase the cytotoxicity of the product, while chitosan and poloxamer 407 may decrease it.

The anti-inflammatory activity of the oromucosal solutions was evaluated using an assay for inhibitory effect on NO production in macrophage RAW 264.7 cell line. The IC<sub>50</sub> values of the oromucosal solutions containing mucoadhesive polymers were not significantly different from those of benzylamine HCl and indomethacin; however, they were approximately twice as high as those of the commercial spray. These results suggest that pharmaceutical excipients in the commercial spray may enhance the inhibitory effect

on NO; whereas, chitosan and poloxamer 407 do not affect the inhibitory effect of benzylamine.

The wound healing activity of the oromucosal solutions was evaluated in the HGF cell line. The results showed that wound healing did not improve when the concentration of benzylamine increased from 1 to 5 µg/mL. However, the solutions containing mucoadhesive polymers demonstrated a statistically significant increase in the rate of mucosal healing compared to the commercial spray. The percentage of wound healing area of the HGF cell line treated with the C0.5P0 solution (benzylamine HCl 1 µg/mL and 5 µg/mL) and its vehicle was more than 90% after 48 hr of the scratch wound creator and closed to 100% after 72 hr. In contrast, the C0P15 solution accelerated wound repair less than the C0.5P0 solution, which may be due to the high viscosities preventing cell migration. These findings support previous studies indicating that chitosan promotes wound healing at different stages of the healing process by enhancing the functions of various inflammatory cells; including polymorphonuclear leukocytes, macrophages, and fibroblasts<sup>21</sup>. Furthermore, chitosan has been found to have good mucoadhesive properties, which can contribute to its effectiveness in promoting wound healing<sup>22</sup>. Additionally, chitosan has been shown to be an effective wound-healing accelerator when combined with other polymers<sup>23</sup>.

The commercial spray exhibited the lowest wound healing activity among all tested solutions at 24 and 48 hr, which may be attributed to the excipients; such as alcohol and the mint flavor in the formulation. Alcohol is widely used in oral care products as a solvent for poorly soluble active ingredients and flavored oils, a penetration enhancer in addition to an active ingredient to control plaque-related oral disease. However, high concentrations of alcohol in the formulation may cause negative effects; such as burning sensations, pain, and dryness<sup>24</sup>. Peppermint oil is popularly used in oral care products to reduce oral malodor and has anti-inflammatory, antimicrobial, and biofilm-inhibiting properties<sup>25,26</sup>. Chlorhexidine at a concentration of 5 µg/

mL induced a cytotoxic effect to HGF cells. Chlorhexidine mouthwash is commonly used to treat oral ulcers and prevent biofilm formation as well as target pathogenic microorganisms in the oral cavity. However, its long-term use may result in reversible discoloration of teeth and mucous membranes, altered taste perception, and serious side effects; such as mouth ulcers and swelling of the salivary glands<sup>27</sup>. Therefore, the use of chlorhexidine at high concentrations and over prolonged periods must be monitored due to its potential toxicity and side effects.

*C. albicans* is an opportunistic pathogen that causes infections in immunosuppressed patients; such as cancer patients undergoing chemotherapy and elderly people with xerostomia, while *S. mutans* is the primary cause of dental caries. The MIC and MFC values of benzylamine against *C. albicans* have been reported to be 250 and 500 µg/mL, respectively<sup>28</sup>. Various MIC values of different chitosans against *S. mutans* and *C. albicans* have been reported in a range of 0.08–5 mg/mL and 0.3–10 mg/mL, respectively<sup>8,29,30</sup>. These differences have been attributed to their molecular weight, degree of deacetylation, pH of the growth medium, and the presence or absence of interfering substances. The antimicrobial action of chitosan has been proposed in different mechanisms. The interaction between positively charged chitosan molecules and negatively charged microbial cell membranes leads to the leakage of proteinaceous and other intracellular constituents as well as altering cell permeability<sup>31</sup>. In this study, the inhibitory activity and microbicidal effect values of chitosan and benzylamine were found to be similar to previous studies. However, there was no data on MBIC and MBEC of benzylamine and chitosan against *S. mutans* and *C. albicans*. The growth of *S. mutans* and *C. albicans* was completely inhibited by C0.5P0 within 1 min, which was as fast as that of a 0.12% w/v chlorhexidine solution, and 180 min, respectively, which was more rapid than with the commercial spray. The reduction in cell number of *S. mutans* and *C. albicans* by longer exposure times of C0.5P15 was found to correspond

to its thermo-responsive property. The antimicrobial activity of benzylamine was found to increase in the presence of chitosan, which is in agreement with previous studies<sup>28</sup>.

## Conclusion

In conclusion, the alcohol-free benzylamine HCl oromucosal solution containing 0.5% w/v chitosan and mild flavoring agents is a promising oromucosal candidate for the treatment of oral mucositis associated with anticancer therapy due to its safety, high wound healing rate, and significant antimicrobial activities.

One notable feature of this solution is its ability to promote wound healing, as evidenced by the complete closure of the scratch wound of the HGF at 72 hr. It was also found to be safer than a commercial spray that contains alcohol and a mint flavor, and a 0.12% chlorhexidine solution. Additionally, the solution demonstrated anti-inflammatory activity in the macrophage raw 264.7 cell line, which was comparable to that of benzylamine HCl. Furthermore, this formulation exhibited antimicrobial and antibiofilm activities against *S. mutans* and *C. albicans* and significantly inhibited the growth of oral pathogens faster than the commercial spray.

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## Conflict of interest

There are no potential conflicts of interest to declare.

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