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RESEARCH ARTICLE

Advancements in Amorphous Solid Dispersions to Improve Bioavailability

Development of a Novel Histatin-5 Mucoadhesive Gel for the Treatment of Oral Mucositis: *In Vitro* Characterization and *In Vivo* Evaluation

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Abstract

Antimicrobial peptides have appeared to be promising candidates for therapeutic purposes due to their broad antimicrobial activity and non-toxicity. Histatin-5 (Hst-5) is a notable salivary antimicrobial peptide that exhibited therapeutic properties in the oral cavity. Oral mucositis is an acute inflammation of the oral cavity, following cancer therapy. The current treatment methods of oral mucositis have low effectiveness. The aim of this study was to design, formulate and characterize a mucoadhesive gel delivery system for Hst-5 usage in the treatment of oral mucositis. Carbopol 934 and hydroxypropyl methylcellulose (HPMC) have been used in the development of a Hst-5 mucoadhesive gel that was optimized by using Box-Behnken design. The optimized formulation was evaluated in-vitro, based on mucoadhesive strength, viscoelasticity, spreadability, release rate, peptide secondary structure analysis, antimicrobial activity, and storage stability. The efficacy of Hst-5 gel was assessed *in vivo* in a chemotherapy-induced mucositis model. The results showed a sustained release of Hst-5 from the new formulation. Hst-5 gel exerted antimicrobial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Candida albicans.* The histopathological, immunohistochemical and statistical analysis showed that the Hst-5 gel had wound healing activity *in vivo*. The findings of this study indicate that the mentioned compound possesses promising potential as a novel and efficient therapeutic agent in managing oral mucositis. Moreover, the results suggest that the compound is commercially feasible for further development and utilization.

Keywords histatin 5 · mucoadhesive gel · mucositis · oral · treatment

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Introduction

Peptides and proteins constitute a significant portion of therapeutics for treating different diseased conditions. This is due to their suitable safety profile, pharmacokinetics, and target specificity [1]. Therapeutic peptides, especially antimicrobial peptides (AMPs), have been shown to harbor multiple biological activities, including anti-inflammatory, anti-microbial, and wound healing effects. AMPs are mostly cationic, amphiphilic, and rich in α-helices [2, 3]. The membrane permeability is recognized as a well-accepted mechanism for describing the mode of action of cationic AMPs [4]. These peptides have unique interactions with microbial cells, making them nontoxic for mammalian cells [5]. Contrary to conventional antimicrobials, AMPs usually do not induce resistance, as they target plasma membrane of

microbial pathogens causing significant changes [6]. This property, as well as their broad range of activities and short contact time required for killing, have made them novel pharmacologic agents [7]. Histatins are cationic antimicrobial peptides, rich in histatin, and are secreted by human salivary glands [8]. Histatin-5 (Hst-5) belongs to the peptides of histatin group, and contains 24 amino acids with anti-bacterial, antifungal and anti-inflammatory activities. It suppresses the production of interleukin 6 and 8 and significantly decreases pro-inflammatory chemokines. In this way, it maintains oral homeostasis and reduces host pro-inflammatory responses [9–11]. Hst-5 also has wound-healing effects and is non-toxic and does not cause drug resistance in the host cells, making it an ideal choice as a therapeutic agent [4, 7]. Oral mucositis is a common and painful condition that is an adverse effect of chemotherapy and radiotherapy. It is characterized by tissue swelling and irritation in the mouth, which can lead to symptoms such as mouth pain, mouth sores, infection, and bleeding [12]. Bacteria and fungi, such as *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli*, and *Candida albicans* have been found with oral mucositis. These microorganisms are known to cause infections and can worsen the symptoms of oral mucositis, such as mouth sores and pain [13, 14]. The current prevention and treatment methods of oral mucositis have low effectiveness, and its management is limited to unsatisfactory symptomatic therapy [15]. Several agents have been studied for preventing or managing oral mucositis and have shown contradictory results. The therapeutic strategies include oral hygiene protocols, anesthetics and analgesics (e.g., morphine, lidocaine), anti-inflammatories (e.g., diphenhydramine, benzydamine, misoprostol), antimicrobial agents (e.g., povidone-iodine, chlorhexidine), cytoprotective agents (e.g., α-tocopherol, glutamine, amifostine, sucralfate) and physical therapies (laser and cryotherapy). These different approaches are frequently used alone or in combination to manage oral mucositis [15]. Meanwhile, very few commercial products exist for relieving the symptoms of oral mucositis. Topical formulations such as Episil® oral liquid, Gelclair®, MuGard® oral mucoadhesive, and Caphosol® have been used with varying results. Therefore, they are not suggested in most guidelines for the management of oral mucositis [16]. A topical drug application is more effective for local action than systemic routes in oral cavity pathologies. Proficient systems for local application can provide a more efficient therapeutic option and reduce drug dosage and adverse side effects [17]. For local treatment of the oral cavity, semisolid or liquid dosage forms are often used. However, the main problem of these traditional systems is their poor retention in the application area, causing suboptimal therapeutic effects. Adding mucoadhesive polymers to dosage forms enhances their retention time and provides physical protection and symptomatic relief for ulcerated oral mucosa. For topical drug delivery, mucoadhesive gels are appropriate as they can provide prolonged release of therapeutic agents to the oral mucosa. Gels are three-dimensional polymers that possess suitable properties which can be modified to design a delivery system with the desired properties for treating oral diseases [18–21]. Some advantages of this dosage form include easy dispersion, painlessness, patient-friendly, and easy self-medication. It also controls the drug release and increases contact with the epithelial surfaces [22]. Mucoadhesive gels have been applied in the oral cavity for the treatment of bacterial and fungal infections [23]. Due to physiological and environmental challenges, the use of peptides and proteins for local action in the oral cavity might be limited. Mucoadhesive polymers help to maintain peptides at the site of application and exert the intended therapeutic effect [15]. The selection of carbopol and HPMC as mucoadhesive polymers is motivated by their ability to improve drug release, and viscosity of the formulation, as well as their ability to sustain drug release and improve mucoadhesive properties [24]. Furthermore, the role of carbopol in protecting proteins and peptides is to alter the velocity of the degradation reaction [25]. Carbopol, with a pKa value of 6.05, swells in an aqueous medium and increases the viscosity of the medium. This swelling inhibits the enzyme from accessing the substrate, thereby reducing enzymatic activity [26]. Carbopol's protective effect on enzymatic degradation has been studied in the context of oral delivery of bioactive proteins and peptides. It has been found to be a useful excipient for this purpose [25].

Although Hst-5 has been shown to have antimicrobial and anti-inflammation activity, there is no literature report about pre-clinical models showing Hst-5's efficacy in the treatment of oral mucositis. Therefore, this study aimed to design, formulate and characterize a mucoadhesive gel containing Hst-5 for investigating its therapeutic application in a rat model of oral mucositis.

Materials and Methods

Materials

Hst-5 peptide was synthesized by the Peptide Synthesis Center of Khajeh Nasir al-Din Tusi University. The method of detecting Hst-5 is mentioned in Supplementary Material. Carbopol 934 and HPMC (viscosity 40–60 centipoise, 2% in H2O), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). Teriadent®, a commercially available oral product (Triamcinolone acetonide 0.1%, Raha Pharmaceutical Co. Tehran, Iran), was used as a positive control. 5-Fluorouracil (5-FU) was purchased from Ebewe Pharma (Ebewe Pharma, Unterach, Austria).

Preparation of Gel

In order to select a suitable polymer for formulation, preliminary studies were carried out. To prepare an Hst-5 gel, the required quantity of carbopol 934 was dispersed in distilled water (DW) which was stirred with a magnetic stirrer. Then, propylene glycol (PG) was added. At the same time, HPMC was dissolved in DW with continuous stirring until a homogeneous solution was obtained. A solution of Histatin-5 was prepared by dissolving the peptide in 1 mM phosphate buffered saline (PBS), after which it was added to the HPMC solution. Histatin-5 was used at a final concentration of 2 mg/ml. Carbopol 934/HPMC blended gel was achieved by adding carbopol 934 solution to the HPMC solution. The formulation was then neutralized by using triethanolamine.

Experimental Design

Design expert® (version 13.0.0) was used to design Box-Behnken for evaluating the effects of variables on the responses and to find the optimal formulation. The variables and their levels were chosen according to preliminary studies. Carbopol 934, HPMC, and PG were selected as independent variables, where mucoadhesive strength, dynamic viscosity (η'), phase angle (Tan δ), and spreadability were designated as responses (Table I). The effect of each independent variable as well as their interaction on each response were determined. The data were fitted to a suitable model via multiple regression, and the best model was chosen for each response, based on ANOVA (P value < 0.05) and the lack of fit with P value > 0.05.

Mucoadhesive Strength of Gels

To measure the mucoadhesive strength of the prepared gels, a modified balance method [27] was applied with some modifications that are mentioned in Supplementary Material.

Table I Variables used in the Box-Behnken Experimental Design

Independent variables	Levels			
	Symbols	−1	0	+1
Carbopol 934	A	0.75	0.88	1
HPMC	B	0.50	0.75	1
PG	C	0	1	2
Responses				
Y1- Mucoadhesive strength	Maximum			
Y2- Dynamic viscosity	Maximum			
Y3- Tan δ	Minimize			
Y4- Spreadability	Maximum			

Viscoelasticity of Gels

In this study, the rheological analysis of formulations was performed using an Anton Par Mcr 301 rheometer (Austria) at different frequencies, ranging from 1 to 10 s^{-1}. For each formulation, oscillatory analysis was performed after determining its linear viscoelastic region at 37 ± 0.1°C, where stress was directly proportional to strain, and the storage modulus remained constant.

Spreadability of Gels

The 'Slip' and 'Drag' characteristics of gels are the basic concepts of spreadability. In this regard, the method [28] was applied with some modifications that are presented in Supplementary Material.

In vitro Release of Hst-5 Gel

To evaluate Hst-5 release profile of the optimal formulation over time, a dialysis bag (D9527 cut-off: 12 kDa) was used [29]. An amount of Hst-5 gel was put into a dialysis bag which was placed in phosphate buffer saline (as dissolution medium, pH 6.8) at 37 ˚C. A magnetic stirrer stirred the buffer at 200 *g*. The samples (each 1 mL) were taken outside the bag at the intervals of 15, 30, 45, 60,120, 180, and 240 min, and each sample was replaced by the same volume of fresh buffer to maintain the sink conditions during the experiment. The amount of released of Hst-5 was determined by the bicinchoninic acid method (BCA) [30]. Then, Hst-5 secondary structure analysis was carried out by Fourier transform infrared spectroscopy (FTIR) and Far-ultraviolet circular dichroism (Far-UV-CD) that are mentioned in Supplementary Material.

The Duration of Adhesion of Hst-5 Gel

The duration of adhesion of Hst-5 gel and Teriadent (as a commercial oral product) were evaluated. In order to measure the duration of adhesion, a method [31, 32] was applied with some modifications. Briefly gel was placed on the upper platform of a test apparatus, and sections of sheep cheek mucus with the mucosal side facing upwards were placed on the lower platform. The apparatus was filled with a phosphate buffer at pH 6.8 and maintained at a temperature of 37ºC. The upper platform was then lowered onto the lower platform, making contact with the mucosal surface of the sheep cheek mucus. After 5 min, a constant tensile stress was applied to the adhesive joint between the gel and the mucosal surface using weights of 2–3.5 g. The digital timer was activated to record the time elapsed until the adhesive

joint detached. The weights were then dropped onto a photocell detector, which automatically stopped the timer and recorded the duration of adhesion of the tested gel.

In Vitro Antimicrobial Evaluation of the Gel

Antimicrobial activities of Hst-5 gel against *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 8739, and *Candida albicans* ATCC 10231 were determined by the agar well diffusion method [33]. The method of determining antimicrobial activities of Hst-5 gel is presented in Supplementary Material.

In Vivo Evaluation of Hst-5 Gel

In order to determine the optimal variables and conditions for animal study (optimal dose, treatment protocol, etc.), preliminary experiments were done, using small groups of animals. To evaluate the efficiency of the optimum formulation in chemotherapy-induced mucositis, 48 male rats (300–350 g, 12–16 weeks) were distributed into four subgroups as follows: G1 group (control), G2 group (gel without Hst-5), G3 group (Teriadent) and G4 group (Hst-5 gel). All rats were weighed at the beginning and the end of the experiment. Oral mucositis was induced by the method [34] that is presented in Supplementary Material. The G1 Group (control) did not receive any treatment. The G2, G3 and G4 Groups received a daily application of gel without Hst-5, Teriadent, and Hst-5 gel respectively, by using flexible swabs. The treatments were applied from the 3rd to 13th day after injury by acetic acid. Histological evaluation for healing was on the basis of Shafer criteria [35]. The animal studies were performed after receiving approval of the Institutional Animal Care and Use Committee (IACUC) in Tehran University of Medical Sciences (IACUC approval No. 1399–1194).

Immunohistochemistry Analysis

Immunohistochemistry analysis for quantifying inflammation, angiogenesis, and soft tissue regeneration (collagen I formation) was performed at the days 7th and 14th. Inflammatory responses were evaluated by CD45 staining, and new blood vessels formation in the wound area was measured by CD31 staining. Collagen deposition was determined by staining for collagen type I. The method of immunohistochemistry analysis is presented in Supplementary Material.

Stability Studies

To evaluate the storage stability, samples of Hst-5 gel were stored for three months under two conditions: $4 \pm 2°C$ (refrigerator) and $25 \pm 2°C$ / $60 \pm 5\%$ humidity [36]. After the first, second, and third months, all samples were assessed for appearance, mucoadhesive force, dynamic viscosity, spreadability, and release rate. Every sample was assessed three times for each storage condition.

Statistical Analysis

Statistical analysis was done, using Sigma Plot version 14.0 (St. Louis, MO, USA). Each experiment was repeated three times and the results were reported as mean ± standard deviation (SD). One-way ANOVA (analysis of variance) test and the Tukey's post-test were carried out. The mean differences were considered significant at P value < 0.05.

Results and Discussion

Optimization of Formulation

A Box-Behnken method was applied to design and optimize the mucoadhesive gel. The concentration of carbopol, HPMC and PG were selected as independent variables. Mucoadhesive strength, dynamic viscosity, Tan δ and spreadability were examined as dependent variables. A total of 15 runs were designed and conducted (Table II). The data were fitted to a suitable model via multiple regression and the best model was chosen for each response, based on ANOVA (P value < 0.05) and the lack of fit with P value > 0.05. The regression equation and the comparative values of R^2 and %CV for each response are given in Supplementary Material (Tables SI to SIV). The three-dimensional plots for each of the four responses mucoadhesive, dynamic viscosity, Tan δ and spreadability, are displayed in Fig. 1. These diagrams are well known for analyzing the interactions between variables and responses. Response surface plots show the effect of carbopol, HPMC and PG concentrations as the main significant variables on four responses.

Mucoadhesive Strength's Measurement of Gels

The mucoadhesive strength of gels was measured by applying a modified method [27] with the results outlined in Table II. According to the results of Table SI, mucoadhesive strength's response is best fitted to a quadratic model ($P < 0.05$) and the model showed precision and significance. Carbopol (A), HPMC (B), and PG (C) were main factors influencing mucoadhesive strength of gels. However, PG had a negative impact on mucoadhesive strength (Table SI). Both factors (A and B) affect mucoadhesive characteristics of gels significantly ($P < 0.05$). As depicted in Fig. 1a, increasing the concentration of carbopol and HPMC enhanced the mucoadhesive strength. This is expected as both polymers are well known for their mucoadhesive properties. The study of Philip *et al*. [37] reported that blends of HPMC

Table II Box-Behnken Design with Results

Run	Cabopol 934 (W/V %)	HPMC (W/V%)	Propylene Glycol (V/V%)	Mucoadhesive strength (N/cm^2)	Dynamic viscosity (pa.s)	Tan δ	Spreadability (g.cm/s)
1	1	0.5	1	5.1	72.76	0.19	6.1
2	0.88	0.5	0	6.35	80.12	0.11	5.6
3	0.75	0.75	2	3.8	48.2	0.25	7.8
4	0.75	1	1	5.5	68.2	0.15	6.5
5	0.88	0.75	1	4.8	60.12	0.19	6.8
6	0.88	1	2	4.1	54.2	0.22	7.2
7	0.88	0.5	2	3.5	49.2	0.28	8.23
8	0.75	0.75	0	6.58	80.5	0.13	5.8
9	0.88	1	0	7.4	85.2	0.14	5.1
10	1	0.75	2	4.1	58.2	0.3	6.5
11	0.88	0.75	1	5.2	65.8	0.19	6.5
12	0.88	0.75	1	5.5	67.2	0.17	6.3
13	1	1	1	6.1	70.2	0.15	5.2
14	1	0.75	0	7.5	88.04	0.12	5.2
15	0.75	0.5	1	4.5	60.5	0.17	7.5

with carbopol 934 were found to have maximum mucoadhesive strength. Chen *et al*. [38] also revealed that the gel containing carbopol with HPMC showed to have suitable mucoadhesive properties and sustained drug release. The study of Hamdi *et al*. [39] found that carbopol 934 and HPMC had better mucoadhesive properties compared to other polymers such as sodium alginate, xanthan gum, and polyvinyl alcohol.

Viscoelasticity Measurement of Gels

In this study, viscoelastic properties of the gels were characterized by dynamic viscosity (η') which reveals flow resistance of the gels. Furthermore, the phase angle (Tan $\delta = G''/G'$) shows relative contribution of viscous components to mechanical properties of the materials used.

The results indicated that dynamic viscosity is best fitted to a quadratic model ($P < 0.05$). The main factors, Carbopol (A), HPMC (B) and PG (C) significantly affect dynamic viscosity. It is observed that both factors A and B have positive effect on the dynamic viscosity of the gel. The negative coefficient for the PG indicated that dynamic viscosity is decreased at higher concentrations of PG. The data of ANOVA analysis of dynamic viscosity are given in Supplementary Material (Table SII). Figure 1b shows that high concentrations of carbopol in the presence of low HPMC concentrations caused a higher dynamic viscosity. This indicates that carbopol is mainly responsible to viscosity of the gels. Overall, the model is significant ($P < 0.05$) and the lack of fit is not significant ($p = 0.3$). The study of Chein *et al*. [38] showed that polymers with higher viscosity usually had better mucoadhesive strength, which was consistent with other studies. Combination of carbopol and HPMC possess a complex structure inducing greater viscosity and better mucoadhesive strength [40–43].

The results showed that phase angle (Tan δ) is best fitted to a quadratic model ($P < 0.05$). The interaction between the main factors is found to be significant ($P < 0.05$). Overall, based on the results of ANOVA, the model is statistically significant ($P < 0.05$) and the lack of fit is not significant ($P = 0.4$). The signal to noise ratio was found to be satisfactory as the observed adequate precision ratio of 20.60 is above 4. The data of ANOVA analysis of phase angle are given in Supplementary Material (Table SIII). As revealed in Fig. 1c, Tan δ was affected considerably by PG concentration in gels, as its increase from 0.5 to 2% caused the rising of Tan δ. The same effect was observed for PG in the presence of carbopol (Fig. 1d). A phase angle smaller than unity indicates the proper structure of the gel because the more the phase angle decreases, the elastic properties increase, and the viscous properties decrease [44]. A large value of Tan δ (G''/G' > 0.1) is typical of so-called weak gels [45]. In this study, the phase angle of the optimal formulation was smaller than unity.

Spreadability Measurement of Gels

The results indicated that spreadability is best fitted to a quadratic model ($P < 0.05$). The data of ANOVA analysis are given in Supplementary Material (Table SIV). The results also revealed that an optimum concentration of polymers was required to achieve the highest spreadability of

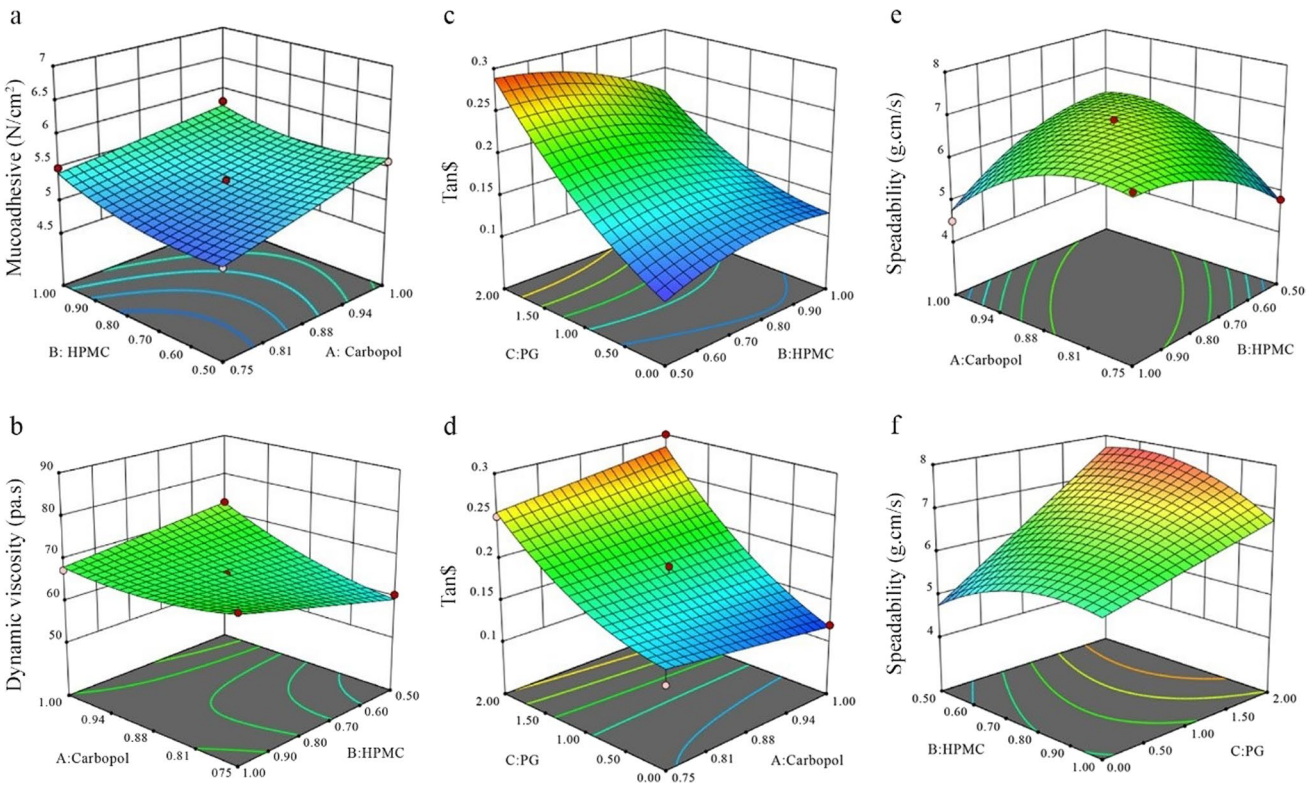

Fig. 1 Response surface plots for four responses: **a** Mucoadhesive strength; **b** Dynamic viscosity; **c** Tan δ (PG, HPMC); **d** Tan δ (PG, Carbopol); **e** Spreadability (Carbopol, HPMC); **f** Spreadability (PG, HPMC)

gels (Fig. 1e). As demonstrated, the middle concentrations of HPMC and Carbopol could produce highest spreading of gels. Also, the results show that PG could significantly increase the spreadability of gels, as its increase from 0.5 to 2% caused about two-fold increase in spreadability of gels (Fig. 1f). It seems that PG as an organic solvent, which modulates the polarity of aqueous medium, can influence the interaction of polymers' chains and consequently their movements in the gels. The spreadability plays an important role in patient's compliance and aids in the uniform application of gel to the skin. A suitable gel takes less time to spread and will have great spreadability [46]. The selection of polymer combinations and relative ratios play a very important role in the development of formulation and should be carefully considered to have appropriate spreadability [47]. The spreadabilty of gels was decreased as the concentration of polymer increased [48]. In this study, the spreadability of the optimal gel formulation was 4.7 ± 0.1 g.cm/s. Our result agree with the study of Swetha *et al*. [49] which formulated a clarithromycin mucoadhesive gel. They reported that the spreadability of the optimal formulation was 4.8 ± 0.1 g.cm/s. In the study by Rasheed *et al*. [50] a polyherbal mucoadhesive gel was prepared and its spreadability was reported 3.4 ± 0.3 g.cm/s.

Validation of the Model

The predicted optimal formulation was composed of carbopol 934, HPMC, and PG, with ratios of 0.9% (w/v), 0.5% (w/v), and 0.6% (v/v), respectively. Table III lists the predicted and observed values of all responses for the formulation. The results showed that the predicted model corresponds to the experimental values in all responses.

Table III The Values of Responses of Optimized Formulation

	Carbopol 934 (W/V %)	HPMC (W/V%)	PG (V/V%)	Mucoadhesive strength (N/cm^2)	Dynamic viscosity (Pa.s)	Tanδ	Spreadability (g.cm/s)
Predict	0.90	0.5	0.6	6.35	87.51	0.11	4.5
Actual	0.90	0.5	0.6	6.45	85.00	0.13	4.7

In Vitro Release of Hst-5 Gel

The cumulative release profile showed that Hst-5 gel has a sustained release with an initial release around 30% in the first 15 min, and by the end of the 4th hour, Hst-5 was completely released (Fig. 2). Chen *et al*. indicated that the gel containing carbopol and HPMC have suitable mucoadhesive properties and sustained drug release[38]. In the study of Pagano *et al*. [51]mucoadhesive-thermoresponsive gel for the treatment of oral mucositis was prepared. They reported a sustained release during 3 h. In a study by Rossi *et al*. [52] benzydamine mucoadhesive gel, consisting of tri methyl chitosan and glycerol phosphate, was made for the treatment of inflammation of mucous. They reported that about 70% of the drug was released during 6 h. In a study by Rezazadeh *et al*. [53] hydrogel containing glycyrrhizin was made for oral mucositis. The drug was released entirely in 3 h. In the study by Ongun *et al*. [54] metronidazole mucoadhesive thermosensitive buccal gel was prepared, using different concentrations of poloxamer 407, poloxamer 188, and HPMC. In their study, the drug release rate was 43% during 8 h. In the study of Venugopal. [55] a silymarin-based mucoadhesive gel for prolonged release in oral mucosa was prepared. They reported that maximum drug release of 96.30% during 3 h. In our study, Hst-5 was entirely released from the gel during 4 h. This release pattern was appropriate for treating mucositis.

Since peptide and protein formulations may react with the carrier due to having positive and negative charges, so an important issue in peptide formulation is to select a suitable carrier that peptide is completely and gradually released. For choosing the best carrier, several polymers were tested. The results showed that for the sustained release of this peptide a combination of carbopol 934 and HPMC was a suitable carrier. Carbopol 934 is a mucoadhesive polymer with high viscosity at low concentrations and toxicity [56]. HPMC, due to its chemical, physical, and biological properties, is mostly applied in the preparation of mucoadhesive gels [57].

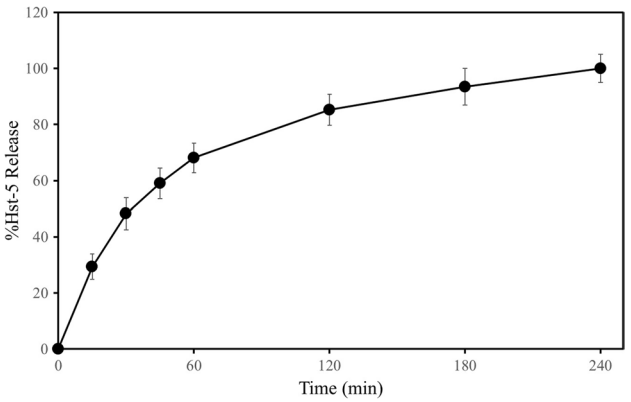

Fig. 2 *In vitro* release profile of Hst-5 from the mucoadhesive gel. Percent of release of Hst-5 from the gel was evaluated at 37°C, using a dialysis bag over 4 h time period. The profile showed that 29.10% ± 3.60% of Hst-5 was released within 15 min and 70.9% ± 4.51% released by 4 h

Table IV Contents of the Secondary Structure of Hst-5, Gels with and without Hst-5

Samples	α- Helix (%)	β-sheet (%)	β-Turn (%)	Random Coil (%)
Gel	0	0	0	0
Hst-5	0	82.9	0	17.1
Hst-5 gel	0	84.0	0	16.0

The FTIR Analysis

To analyze the secondary structure of Hst-5 before/after the formulation, an FTIR study was carried out. The spectra of the gel in the presence and absence of Hst-5 were recorded within 4500 cm^{-1} to 500 cm^{-1}(Fig. 3a). The Hst-5 spectrum showed characteristic bands of amide I (between 1600 and 1700 cm^{-1}) and also amide A (between 3400 and 3500 cm^{-1}) that are directly related to their backbone conformation. Several studies concluded that amide I, corresponding to the β-sheet in peptides, was observed at 1600—1700 cm^{-1} [58–60].The presence of the amide I band before and after formulation revealed that the secondary structure of Hst-5 after formulation was conserved. The IR Spectra showed no incompatibility between Hst-5 and polymers. The FTIR study indicated that Hst-5 was successfully loaded into a combination of carbopol and HPMC.

The CD Analysis

Circular Dichroism (CD) spectroscopy was carried out to provide information about the secondary structure of Hst-5, before and after the formulation. The CD spectrum of the Hst-5 in 100 mM phosphate buffer saline (pH 6.5) at 20°C indicated a characteristic β-sheet structure with negative peaks around 182 – 185 nm (Fig. 3b). The CD spectra analysis, using the CDNN (Circular Dichroism analysis using Neural Networks) program, confirmed the obtained results (Table IV). According to these results, β-sheet and random coil in the Hst-5 alone and the Hst-5 in the formulation were not significantly changed, which indicates that the secondary structure of the peptide is preserved during formulation.

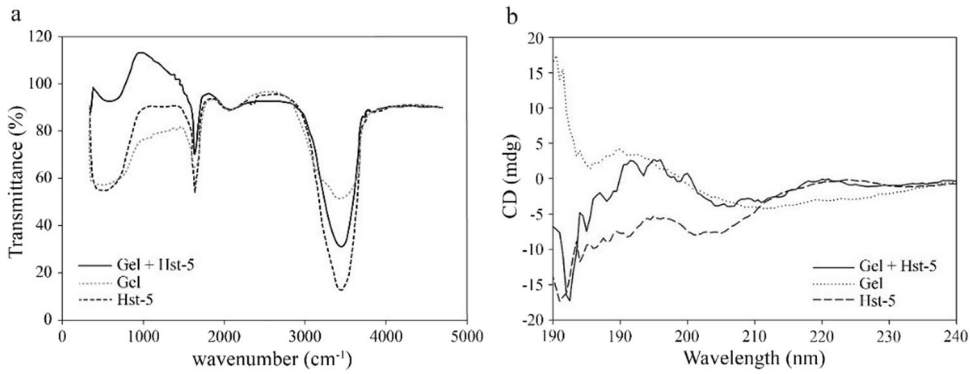

Fig. 3 **a** FTIR was carried out using IR spectroscopy (Shimadzu, Japan). The spectra of the Hst-5 (dash line), the gel without Hst-5 (dotted line), and the Hst-5 gel (solid line) were recorded within 4500 cm^{-1} to 500 cm^{-1}. **b** Far-UV-CD spectroscopy (Aviv 215 USA) at 20°C from 180 to 240 nm, with the interval of 1 nm was applied to analyze the peptide's secondary structure before/after the formulation. Far-UV-CD spectra of the Hst-5 gel (solid line), gel without Hst-5 (dotted line) and Hst-5 (dash line)

The Duration of Adhesion of the Optimum Formulation

The results showed that with the application of 2g weight, Hst-5 gel was observed to have remained adhesive on the mucosal surface for a period of 23 h (SD = 1.3) and for Teriadent was 22.5 h (SD = 1.5). Increasing the weight applied to Hst-5 gel and Teriadent lead to the shortening of duration of adhesion (Fig. 4). The retention time of a mucoadhesive dosage form at its area of attachment would be expected to depend on the rate of the forces to which it is subjected. The results showed that there were no significant differences in duration of adhesion of Hst-5 gel and Teriadent (*P* value > 0.05, Student's t-test).

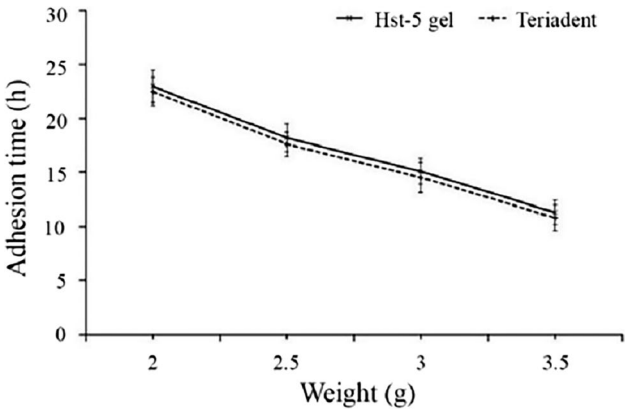

Fig. 4 The effect of varying the applied weights (2, 2.5, 3 and 3.5 g) on the duration of adhesion of Hst-5 gel (solid line) and Teriadent (dash line) to sheep's cheek mucosa in pH 6.8 phosphate buffer. There were no significant differences in the duration of adhesion of Hst-5 gel and Teriadent (*P* value > 0.05 Student's t-test). In both Hst-5 gel and Teriadent, increasing the applied weight shortened the duration of adhesion

Antimicrobial Activity of Hst-5 Gel

The results of the zone of inhibition for *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, and Candida albicans* are reported in the Table V. Values are shown as the average of three replicates ± standard deviation. Based on the literature [33], if the zone of inhibition is 5–10 mm, 10–19 mm, or ≥ 20 mm, the inhibition activity is moderate, strong, and excellent, respectively. Results indicated that Hst-5 gel possesses strong activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa, Escherichia coli, and Candida albicans*, and has excellent activity against *C. albicans.*

In Vivo Efficacy Evaluation

A chemotherapy-induced oral mucositis model was used to assess the *in vivo* efficacy of Hst-5 gel. On the seventh day, H&E staining results indicated that the G1 group (control) exhibited ulceration covered with necrotic area and micro-abscess formation and there was no re-epithelialization. The G2 group (gel without Hst-5) showed severe infiltration of inflammatory cells and re-epithelialization was not present. In the G3 group (Teriadent), epithelial proliferation in the margin of ulcer was seen and inflammatory cells were decreased compared to the G1 and G2 groups. The G4 group (Hst-5 gel) had re-epithelialization, granulation tissue formation, and marked fibroblast proliferation in underlying stroma (Fig. 5a). On the fourteenth day, in the G1 group (control) basal membrane had lost its integrity and also chronic inflammatory infiltrate was seen. In the G2 group (gel without Hst-5) inflammation was still obvious and there was no sign of re-epithelialization. In the G3 group (Teriadent), granulation tissue was formed and mild inflammatory infiltrate was present. The G4 group (Hst-5

Table V *In vitro* Antimicrobial Activity of the Hst-5 gel Against Standard Antimicrobial Agents. Values are Averages of Three Replicates ± Standard Deviation (Mean ± SD)

Microorganism	Hst-5 gel (mm)	Ciprofloxacin (mm)	Nystatin (mm)
Staphylococcus aureus	18 ± 1.2	20 ± 1.5	-
Pseudomonas aeruginosa	15 ± 1.5	18 ± 1.3	-
Escherichia coli	13 ± 1.3	16 ± 1.5	-
Candida albicans	21 ± 1.1	-	23 ± 1.2

gel) showed full re-epithelialization with more blood vessels. Furthermore, connective tissue revealed normal features and granulation tissue with fibroblasts (Fig. 5b). Also, Masson's trichrome staining releaved that many newborn collagen fibers appeared in the G4 group (Hst-5 gel) in comparison to the other three groups (Fig. 5c). Recent studies suggest that high inflammatory cells in the wound area are related with exacerbation of wounds [61]. Angiogenesis, the formation of new blood vessels, is important for wound healing. At the initial of the healing process, vesseles growth is ferocious, because of increasing demand for oxygen, nutrients, and immune cells in the wound site [62]. In this study, Inflammatory responses were evaluated by CD45 staining, and CD31 staining was done to check the density of blood vessels in the wound area. Collagen deposition was assessed by staining for collagen type I. On the seventh day in the G1 group (control), a few number of CD31 marker were present. The data of the number of CD31 on the 7th day are given in Supplementary Material (Table SV and SVI). The marker CD45 was increased, which indicated a high level of inflammatory cells in the area of wound (Table SVII and SVIII). In this group, the expression of collagen I revealed to be very few (Table SIX and SX). The G2 group (gel without Hst-5) had a few numbers of CD31 marker, which showed the formation of few blood vessels in this group. The CD45 marker was elevated and few collagens I was seen (Table SV to SX). In the G3 group (Teriadent), expression of CD31 marker was increased relative to the G1 and G2 groups. The CD45 marker was decreased compared to the control and the gel without Hst-5 groups, and the marker collagen I was expressed more than G1 and G2 groups (Table SV to SX). In the G4 group (Hst-5 gel), expression of CD31 marker was increased in comparison to the other three groups. The CD45 marker decreased relative to the G1, G2 and G3 groups, and collagen I was expressed more than the other three groups. The data are given in Supplementary Material (Table SV to SX). On the 14th day, the CD31 marker showed an increase in the G4 group (Hst-5 gel) relative to the G1, G2 and G3 groups, which indicated the formation of more matured vessels in G4 group (Fig. 6a, Table SV and SVI). The CD45 marker was significantly decreased in the G4 group compared to the other three groups, and that revealed considerable reduction in inflammatory cell infiltration in the wound area in the G4 group (Fig. 6b, Table SVII and SVIII). The

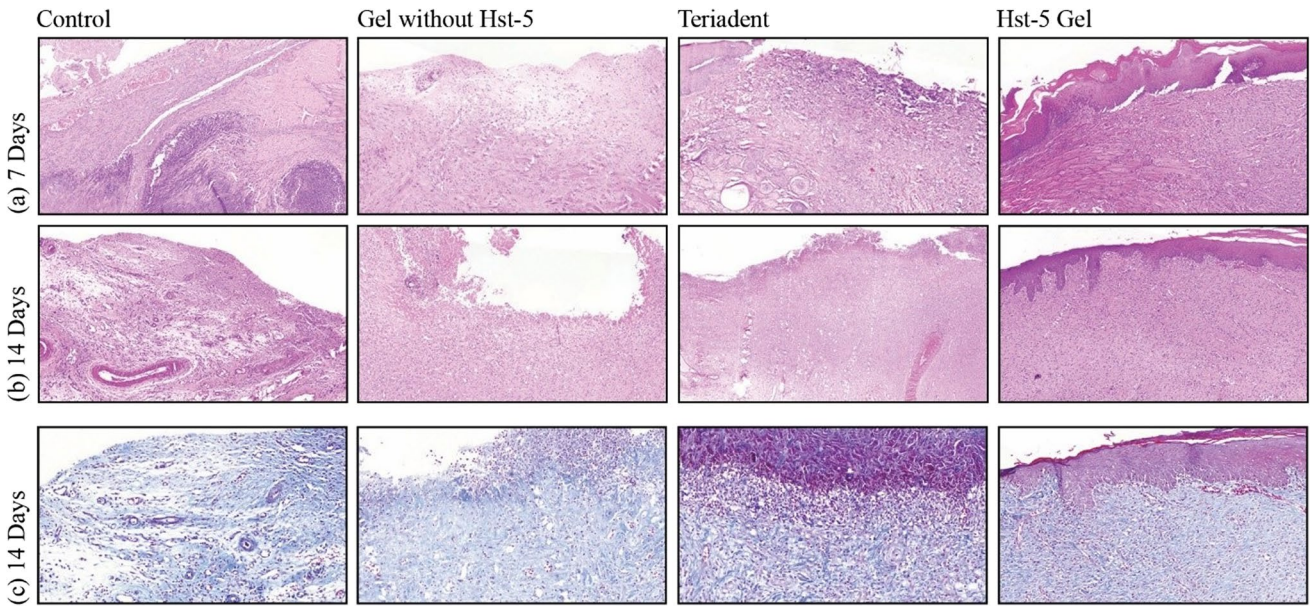

Fig. 5 Representative histopathology images of Hematoxylin–eosin staining of the cheek mucosa of rats in the four groups: Control (no treatment), gel without Hst-5, Teriadent and Hst-5 gel **(a)** Microscopic aspects of these groups on the day 7th of experimentation. Magnification 4x, **(b)** Microscopic aspects of groups on day 14th of experimentation. Magnification 4x, **(c)** Representative histopathology images of Masson's trichrome staining of the cheek mucosa of rats in the four groups on the day 14th of experimentation. Magnification 10x

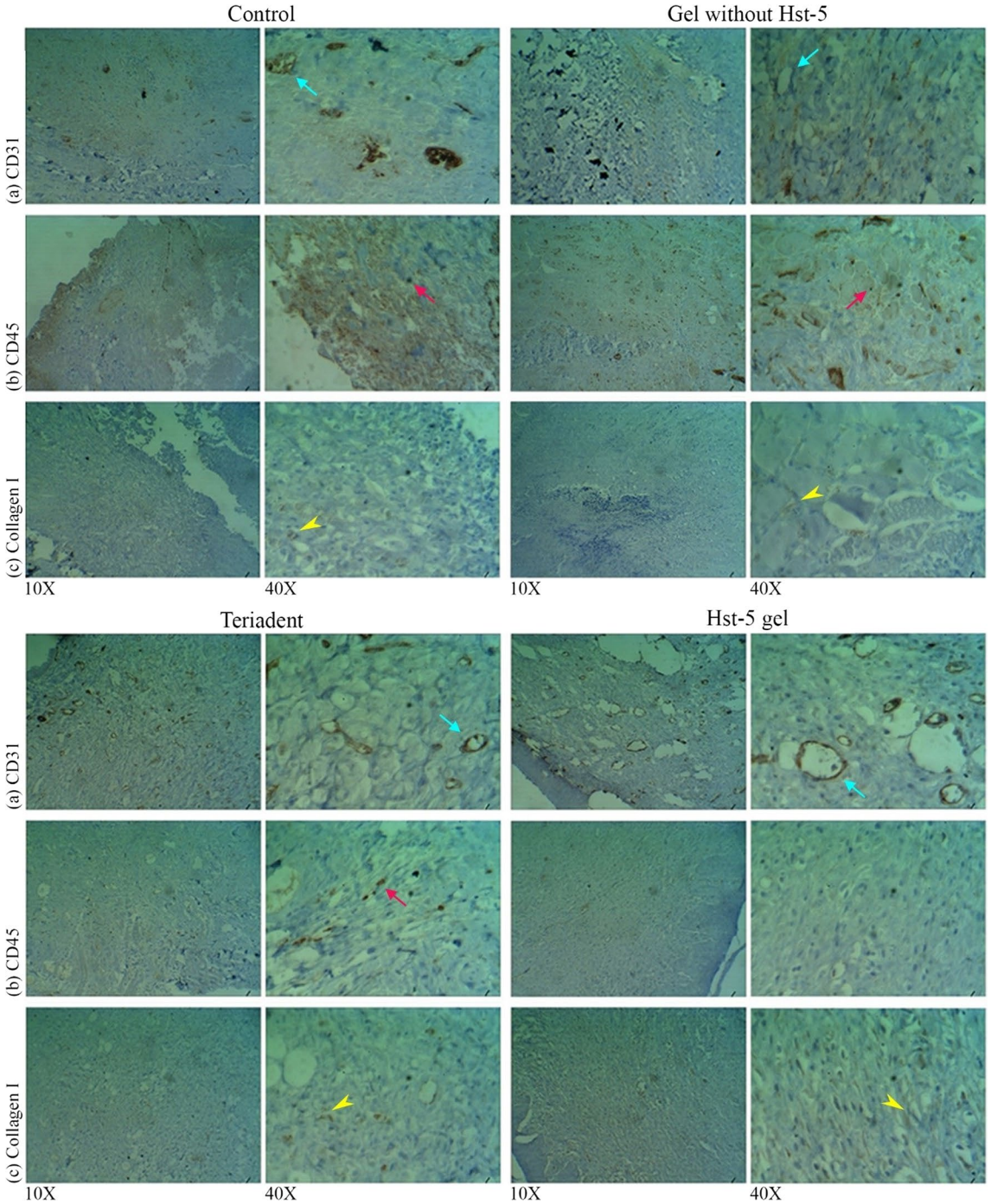

Fig. 6 Immunohistochemical images of cheek mucosa of four groups: Control (no treatment), gel without Hst-5, Teriadent and Hst-5 gel on the day 14th of experimentation **a)** CD31 (new blood vessels), **(b)** CD45 (inflammatory cells), **(c)** collagen I. Light blue arrow; blood vessels, red arrow; inflammatory cells, yellow arrowhead; collagen. Magnification 10 × and 40x

expression of collagen I was increased in the G4 group relative to other three groups (Fig. 6c, Table SIX and SX).

Overall, On the seventh and fourteenth days, the G4 group (Hst-5 gel) had statistically significant difference from the other three groups in terms of CD45 (inflammatory cells) count reduction (Fig. 7a), increase in CD31 (new blood vessels) count (Fig. 7b), and collagen I formation (Fig. 7c). Our results showed that Hst-5 gel could decrease the number of inflammatory cells in the wound area and confirmed good healing activity in the treatment of mucositis. This was due to the healing effect of Hst-5 which promoted more fibroblasts formation with

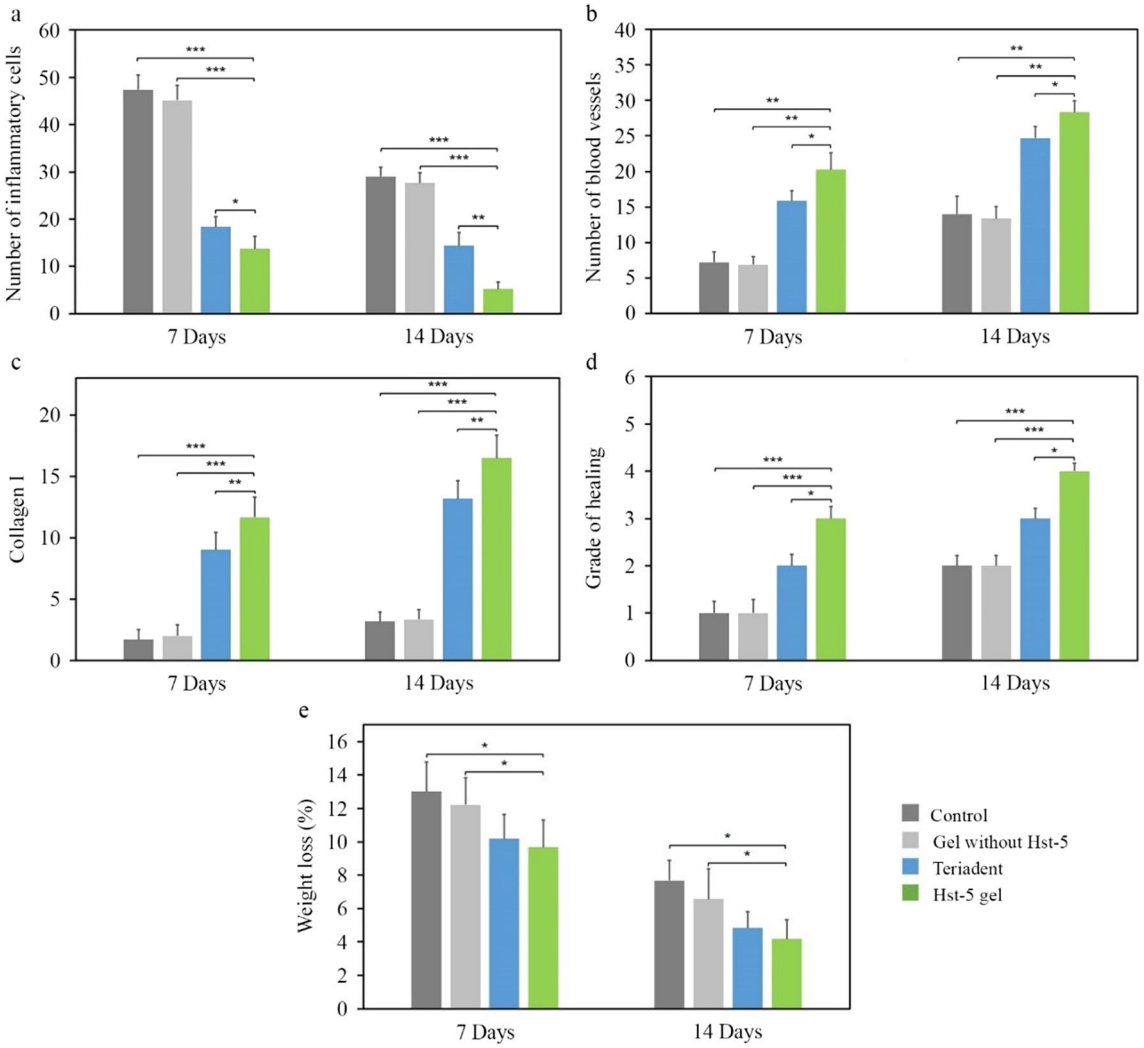

Fig. 7 **a** Number of Inflammatory cells (CD45 marker) **b** Number of new blood vessels (CD31 marker) **c** Percentage of collagen (collagen I marker) **d** Grade of healing **e** Percentage of weight loss according to the group and the day of experimentation. Hst-5 gel *vs.* gel without Hst-5, Teriadent and control groups on the day 7th and 14th of experimentation (One-Way Analysis of Variance, followed by Tukey's post-test *P*-values indicate statistical significance (*$P<0.05$, ** $P<0.01$, *** $P<0.001$)

more collagen in local lesion. The G4 group (Hst-5 gel) had statistically significant difference from the other three groups in terms of grade of healing on the seventh and fourteenth days (Fig. 7d). All animals lost weight throughout the experiment due to the side effects of 5-fluorouracil, oral mucositis, and reduction in eating as well as drinking water. The Hst-5 gel group showed a lower percentage of weight loss compared to the other three groups on the seventh and fourteenth days (Fig. 7e). The study of Rajaee *et al*. [63] showed that doxepin's gel had a slow release from the formulation, which could heal inflammations of the oral mucosa in cancer patients, although applying topical doxepin may have some side effects, such as allergic reaction, dry mouth, and burning at the site of application [64]. Vladimir *et al*. [65] showed that mucoadhesive propolis gel could be a suitable topical formulation for preventing oral mucositis. Meanwhile, propolis may have some side effects, such as irritation in application, mouth sores, lesions, or eczema [66]. The study of Ongunt *et al*. [54] indicated that mucoadhesive gel containing metronidazole might be a good option in treating oral mucositis. However, metronidazole gel causes burning, skin irritation, dryness, and redness [67]. The Hst-5 has many advantages over other therapeutic materials mentioned above. It possesses broad-spectrum antibacterial activities, antifungal, and anti-inflammation effects during wound healing. Furthermore, Hst-5 gel creates a physical protecting barrier against external factors by covering the inflamed oral mucosa. Moreover, it has a long residence time at the site

Table VI Results of Stability Studies of the Hst-5 Gel. Values are Averages of Three Replicates ± Standard Deviation (Mean ± SD)

Time	Temp	Mucoadhesive strength (N/cm^2)	Dynamic viscosity (Pa.s)	Spreadability (g.cm/s)	Hst-5 content release (%)
0	-	6.5 ± 0.2	84.74 ± 1.2	4.7 ± 0.1	99.80 ± 1.3
1 Month	4	6.4 ± 0.1	85.00 ± 1.2	4.5 ± 0.3	99.64 ± 1.5
	25	6.3 ± 0.2	83.57 ± 1.5	4.35 ± 0.6	94.75 ± 2.2
2 Months	4	6.2 ± 0.2	83.4 ± 1.7	4.3 ± 0.2	99.45 ± 1.5
	25	6.25 ± 0.4	82.20 ± 1.6	4.25 ± 0.5	83.64 ± 2.5
3 Months	4	6.15 ± 0.1	82.5 ± 2.3	4.2 ± 0.1	99.38 ± 1.2
	25	6.0 ± 0.3	80.67 ± 2.5	4.15 ± 0.3	75.67 ± 1.6

of application and sustained release and so accelerates the healing of mucosal lesions.

Stability Studies

The gel samples were assessed on the basis of appearance, mucoadhesive strength, dynamic viscosity, spreadability, and the release of the peptide at the time of preparation (before storage) and for the storage at 4°C and 25°C (1, 2, and 3 months post-preparation).The gels did not show any color changes during the storage period. The results are reported in Table VI. Stability studies showed that these formulations were almost stable for up to three months in two storage temperature (4°C and 25°C) but at 25°C the release of Hst-5 from the gel was decreased.

Conclusion

It is challenging to make a peptide formulation for local application in the oral cavity due to its small size and instability. Thus, to preserve the therapeutic properties of peptides, it is required to have suitable excipients in the formulation to achieve effective remedies. In the present study, Hst-5 gel was prepared based on carbopol 934 and HPMC, and the formulation was optimized, using a Box-Behnken design. The optimal formulation revealed desirable mucoadhesive strength, viscoelasticity, and spreadability. Furthermore, it possessed sustained release and an appropriate residence time in the oral mucosa. The results of FTIR and CD indicated that the structure of the peptide in our formulation is persevered. Moreover, Hst-5 gel showed antimicrobial activity against *S. aureus*, *E. coli*, *P. aeruginosa*, and *C. albicans*. The findings of this study demonstrated that Hst-5 gel is a *stable formulation* with wound healing activity *in vivo*. Thus, the formulation showed to be a promising candidate for the treatment of oral mucositis.

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Author Contribution Shiva Golshani: Conceptualization, investigation, visualization, methodology, performed experiments, data analysis, and wrote the manuscript. Alireza Vatanara: Supervision, conceptualization, methodology, visualization, reviewing and editing. Saeed Balalaie: Participated and assisted in experiments and conceptualization. Zeinab Kadkhoda: Participated and assisted in experiments and conceptualization. Mohammad Abdollahi: Data analysis, visualization, reviewing and editing. Mohsen Amin: Supervision, conceptualization, methodology, resources, reviewing and editing.

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Data Availability The datasets supporting study are available from the corresponding author on reasonable request.

Declarations

Competing Interest The authors declare no competing interests.

Conflict of Interest The authors declare no conflict of interest.

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