Journal of Research in Pharmacy

Research Article

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Natural ingredients included antimicrobial lozenge formulations for oral care

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Received: 10 May 2023 / Revised: 09 July 2023 / Accepted: 13 July 2023

ABSTRACT: Lozenges are easy-to-use solid/semi-solid dosage forms that can contain one or more active ingredients, which can be used in the local treatment of mouth and throat infections. Honey, propolis, and perga, which are bee hive natural products have properties of antimicrobial and nutrient. While the oil of seaweed has a rich omega-3 content, the essential oils of pine turpentine, juniper, eucalyptus, sage, lemon balm, and tea tree have antimicrobial properties. In this study, it is aimed to prepare the above indicated ingredients included antimicrobial lozenges. Prepared formulations were evaluated by characterization and antimicrobial efficiency tests. Characterization tests included the determination of organoleptic parameters, weight variation, friability, and in vitro disintegration time. Antimicrobial efficacy was determined with the antimicrobial activities of lozenges against group A β-hemolytic *streptococci*, group B β-hemolytic *streptococci*, *Staphylococcus aureus*, *Klebsiella pneumoniae,* and *Candida albicans* which was evaluated by broth microdilution and time-kill studies. Results showed that all the formulations showed acceptable characterization profiles and antimicrobial efficiency. The lozenge formulation, which included honey, propolis, Perga, seaweed oil, and essential oils of pine turpentine, juniper, and eucalyptus can be accepted as the best formulation in terms of antimicrobial activities against all of the tested microorganisms especially Group A beta-hemolytic streptococci which is the most frequent bacterial pathogen causing sore throat in the oral cavity. In conclusion, natural ingredients included lozenges can be a good alternative for oral care products with their good antimicrobial properties for a wide range of populations.

KEYWORDS: Honeybee products; essential oils; lozenge; oral care

1. INTRODUCTION

The oral cavity has a very complicated microbiota containing around 700 different microorganisms. The type of oral microflora that can survive is determined by unique characteristics of the oral cavity such as the surface of the tongue and epithelium, the physiological factors such as pH, temperature, nutrients, redox potential, and gingival crevicular fluid. When the balance in the composition of oral microbiota alters due to the use of antibiotics, lack of hygiene, inappropriate diets such as frequent use of fermentable carbohydrates, immunosuppression, oral surgery, and trauma, it may cause oromucosal, buccal, dental and throat infections [1,2]. Opportunistic pathogens, such as *Staphylococcus aureus* (Gram-positive bacteria), *Klebsiella pneumoniae* (Gram-negative respiratory bacteria), group A β-hemolytic *streptococci* and group B β-hemolytic *streptococci* (Gram-positive bacteria) and *Candida albicans* (yeast) may cause a variety of infections in the mouth or throat such as sore throat, oral mucositis and spread to the respiratory system, lower digestive tract, and, after crossing the blood-brain barrier, the nervous system of the host [2,3,4,5]. Oral care is considered one of the most important prophylactic approaches for the maintenance of oral health and consequently the rest of the human organism [6].

Oral health could be maintained using various products in the forms of, for example, gels, liquids or lozenges. Lozenges are flavored solid dosage forms and are generally used to relieve oral/oropharyngeal local infections and related symptoms. They are also widely used as a support in the treatment of upper respiratory tract infections or as a food supplement for children. They can reach a large population all over the world due to their easy preparation and low cost, as well as the fact that it is intended for the use of individuals from all age groups. Natural ingredients-based lozenges are gaining popularity due to the various pharmacological

How to cite this article: Esentürk-Güzel İ, Abdo L, Topuzoğlu S, Yıldız C, Yılmaz F, Döşler S. Natural ingredients included antimicrobial lozenge formulations for oral care. J Res Pharm. 2024; 28(1): 248-257.

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ISSN: 2630-6344

http://dx.doi.org/10.29228/jrp.692

activities of the molecules found in their structure. Honey bees' products, herbal extracts and essential oils are frequently preferred in recent years to promote good balance in oral flora and oral hygiene for healthier mouth and throat [1,7,8,9,10]. In this direction, it could be beneficial to increase the diversity of use of the lozenges with novel formulations including natural substances.

Honey bees' hive main products are honey, propolis and Perga. Honey is a naturally occurring supersaturated sugar solution and is mostly used as a sweetener and also contains many bioactive components which provide antioxidant, antibacterial, anti-inflammatory, and wound healing properties [8]. Propolis is a resinous substance, which contains compounds with high antimicrobial and antioxidant is collected and processed by honey bees (*Apis mellifera*) from cracks in the leaves, flower buds, stems and bark of many tree species [11]. Bee bread with its other name Perga is the fermentation of bee pollen mixed with bee saliva and flower nectar inside the honeycomb cells of a hive, which has several nutritional virtues and various bioactive molecules with curative or preventive effects [12]. Oil of seaweed (*Chondrus crispus*) has a rich omega 3 content [13] and essential oils of pine turpentine (*Pinus spp.*) [14], juniper (*Juniperus communis*) [15], eucalyptus (*Eucalyptus globus L.*) [16], sage (*Salvia officinalis L.*) [17], lemon balm (*Melissa officinalis L.*) [18] and tea tree (*Melaleuca Alternifolia L.*) [19] have antimicrobial properties. Among these, pine turpentine oil has strong antibacterial, antifungal, antiseptic, antiviral, analgesic, and anti-inflammatory activities [20]. Juniper oil also has strong antiseptic, antibacterial and antifungal activities [15]. The other above-indicated essential oils have mild antimicrobial activities and are generally used for their flavoring properties [19,21,22,23]. In the literature, there is not any study of lozenge formulations containing above mentioned natural sources together. Considering these properties of those natural ingredients, lozenge formulations of the above sources combination, which have antimicrobial properties in the mouth for oral care aimed to be prepared. As a preliminary study, a lozenge formulation included honey, propolis (water-based), Perga and essential oil of *Syzygium aromaticum* were prepared and evaluated by antimicrobial tests [24].

In this study, various natural ingredients included four different lozenge formulations were prepared. Honey, propolis, Perga, pine turpentine and juniper essential oils were used for their strong antimicrobial properties and seaweed oil was used for its omega-3 content in all formulations. Additionally, the lozenge formulations included either the essential oils of eucalyptus, sage, lemon balm or tea tree for their antimicrobial and flavoring properties. The aim of preparing four different formulations was to obtain a synergistic antimicrobial and flavoring effect by using the essential oils of eucalyptus, sage, lemon balm or tea tree in combination with the other various strong antimicrobial ingredients. The prepared lozenges were then characterized by the determination of organoleptic parameters, weight variation, friability and in vitro disintegration time. Antimicrobial efficacy was determined with the antimicrobial activity of lozenges broth microdilution and time-kill studies against group A β-hemolytic *streptococci*, group B β-hemolytic *streptococci*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Candida albicans* strains.

2. RESULTS AND DISCUSSION

2.1. Preparation of lozenges

Four lozenge formulations from natural ingredients combinations were prepared successfully by the moulding method. All of the lozenges were easily removed from the molds and were good in shape. The photographs of the prepared lozenges were given in Figure 1.

2.2. Characterization of lozenges

2.2.1. Organoleptic properties

Organoleptic properties are important to have good patient compliance. The prepared lozenges were examined in terms of taste, odor, color, softness, shape and surface smoothness properties. The organoleptic properties for all lozenge formulations were at a level acceptable to the patient. The organoleptic properties of the prepared lozenge formulations were given in Table 1.

2.2.2. Weight variation, diameter, thickness, friability and in vitro disintegration time

The results of the studies for the evaluation of weight variation, diameter, thickness, friability, and in vitro disintegration time of the lozenge formulations were given in Table 2. The mean values of weight, diameter and thickness of the lozenge formulations were between 0.62 g-0.90 g, 14.65 mm-15.57 mm and 3.06 mm-4.11 mm, respectively, which were all suitable for oral application. According to the USP standard, the weight variation of the lozenges should not be more than 5% [25]. Given that, all of the formulations were slightly higher than the limit. The percentage friability value of the lozenges should not be more than 1%

[26,27]. According to the results, all lozenge formulations had low friability values, indicating suitability for packaging [28].

The organoleptic and slow disintegrating properties of the lozenge dosage forms differentiate them from traditional tablets [29]. *In vitro* disintegration time is defined as the duration needed for the complete disappearance of the lozenge from the tester net [30]. According to the results, the mean disintegration time was found to be between 5 min 7 s ± 28 s and 6 min 39 s ± 46 s, which could be considered as encountering the requirements for an oral care product [30].

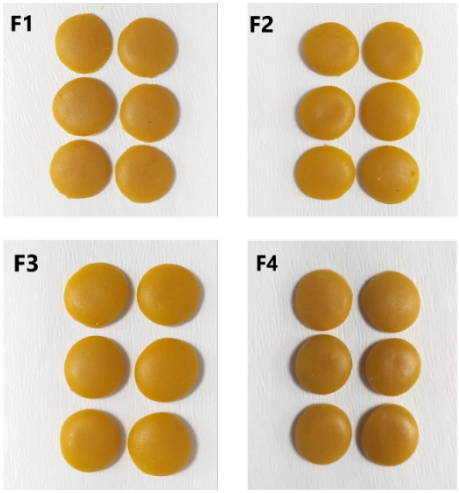

Figure 1. The photographs of the prepared lozenge formulations

2.3. In vitro antimicrobial activity

2.3.1. Determination of minimum inhibitor concentrations (MIC)

According to the results obtained by determining the MICs, all lozenge formulations (dissolved in 10 ml of artificial saliva liquid) were found to be effective against all tested mouth-throat and upper respiratory tract infections causing microorganisms at the concentrations ranging from direct-1/4, usually at 1/2 dilution. Additionally, among the lozenges, F1 could be selected as the more active formulation against all of the microorganisms (at 1/4 or 1/2 dilution). Group A beta-hemolytic *streptococci* is the most frequent bacterial pathogen causing acute pharyngitis (sore throat), which is the second most common acute infection seen by family doctors. Group A beta-hemolytic streptococci are responsible for 5% of pharyngitis in adults and 20% of pharyngitis in children [31,32]. Also, *Candida albicans* is one of the most abundant pathogenic microorganisms found in oral microbiota and may cause oral candidiasis [2]. Considering that, F1 is the most active formulation against group A beta-hemolytic *streptococci* and F2 is the most active formulation against *Candida albicans* both at 1/4 dilutions.

While the oral fluid tested for control did not have any antimicrobial activity, the MIC values of standard antibiotics and antifungal agents used for the standardization of the experiment were within the quality control limits determined by CLSI. The results of the study of the determination of minimum inhibitory concentrations of the lozenges were given in Table 3.

Table 1. Organoleptic properties of the lozenge formulations.

Organoleptic properties	Formulation Number			
	F1	**F2**	**F3**	**F4**
Taste	Fresh and sweet	Sweet	Sweet	Light and sweet
Odor	Mild honey	Sage	Mild melissa	Mild honey

Color	Gold	Gold	Gold	Gold
Softness	Slightly hard	Slightly hard	Slightly hard	Slightly hard
Shape	Round	Round	Round	Round
Surface smoothness	Smooth	Smooth	Smooth	Smooth

Table 2. Weight variation, diameter, thickness, friability and *in vitro* disintegration time values of the lozenge formulations.

Parameters	**Formulation Number**			
	F1	**F2**	**F3**	**F4**
Weight variation (g)	0.68 ± 0.10	0.62 ± 0.06	0.90 ± 0.14	0.87 ± 0.12
Diameter (mm)	15.57 ± 0.49	14.89 ± 0.79	14.65 ± 1.06	15.02 ± 0.68
Thickness (mm)	3.06 ± 0.33	3.14 ± 0.27	4.11 ± 0.37	4.00 ± 0.46
Friability (%)	0.06	0.08	0.01	0.04
In vitro disintegration time	5 min 27 s ± 1 min 18 s	5 min 7 s ± 28 s	6 min 16 s ± 1 min 12 s	6 min 39 s ± 46 s

2.3.2. Time kill curve (TKC) study

According to the results obtained by the time kill study, the activity of the lozenges against all tested mouth-throat and upper respiratory tract infection-causing microorganisms started rapidly and showed activity with an average killing rate of 2 Log10 compared to the initial number between 30 minutes and the first hour. In the second hour, regrowth was seen, and the starting number was approached. An average of 2-3 Log10 differences was observed for the lozenge formulations at all times compared to the control (artificial saliva) which was not treated with any substance. The graphs of the time-kill curve study of the lozenges were given in Figure 2.

Table 3. Minimum inhibitory concentrations (MICs) of the lozenge formulations.

Formulation	**Strains**				
	AGBHS, Clinical strain	**BGBHS, Clinical strain**	***S. aureus* ATCC 29213**	***K. pneumoniae* ATCC 4352**	***C. albicans* ATCC 10231**
F1	1/4	1/2	1/2	1/2	1/2
F2	1/2	1/2	Direct	1/2	1/4
F3	1/2	1/2	Direct	1/2	1/2
F4	1/2	Direct	Direct	1/2	1/2

Artificial saliva	-	-	-	-	-
Ciprofloxacin	1	1	0.25 µg/ml	0.5 µg/ml	-
Fluconazole	-	-	-	-	2

(The dilutions shown in the table means the highest dilution (lowest concentration) at which the lozenges can exhibit antimicrobial activity compared to direct use.)

3. CONCLUSION

Good oral hygiene supports prevent infections in the mouth or throat caused by opportunistic pathogens such as *Staphylococcus aureus*, *Klebsiella Pneumoniae*, group A β-hemolytic *streptococci* and group B β-hemolytic *streptococci* and *Candida albicans* and thus oral care is the most important measure to achieve oral and eventually overall health. Lozenges are easy use and prepared solid/semi-solid dosage forms that can contain one or more active ingredients, which can be used in local treatment of oral infections. Today, natural ingredients-based lozenges have gained attraction and lozenge formulations can be enriched using these substances. Using honey bees' products, herbal extracts and essential oils contributes to oral hygiene for a healtier mouth and throat. As in our study, honey bee hive products, seaweed oil and essential oils are good alternatives in terms of their nutrient, flavoring and antimicrobial properties. The essential oils of either eucalyptus, sage, lemon balm, or tea tree contributed to preparing good lozenge formulations which have mild to strong antimicrobial activities. Organoleptic characteristics of all the prepared lozenges achieved the required patient standards, especially in terms of their sweet, fresh or light sweet taste. All of the lozenges disintegrated in artificial saliva between 5-6 min, which was suitable for oral care products. Moreover, all lozenge formulations were found to be effective against all tested mouth-throat and upper respiratory tract infections causing pathogens. Their efficacy started after dissolving in the mouth and reached to maximum between 30 minutes and the first hour according to the time kill study, which is important to have a fast action against the microorganisms. In addition, among the lozenges F1 formulation, which differs from other formulations by including eucalyptus essential oil, could be chosen as the best formulation in terms of high antimicrobial activities against all of the tested opportunistic microorganisms especially Group A beta-hemolytic *streptococci* which is the most frequent bacterial pathogen causing sore throat in the oral cavity. In conclusion, natural ingredients such as honey bee hive products, seaweed oil and essential oils included lozenges can be a good alternative for oral care products with their good antimicrobial properties for a wide range of the population.

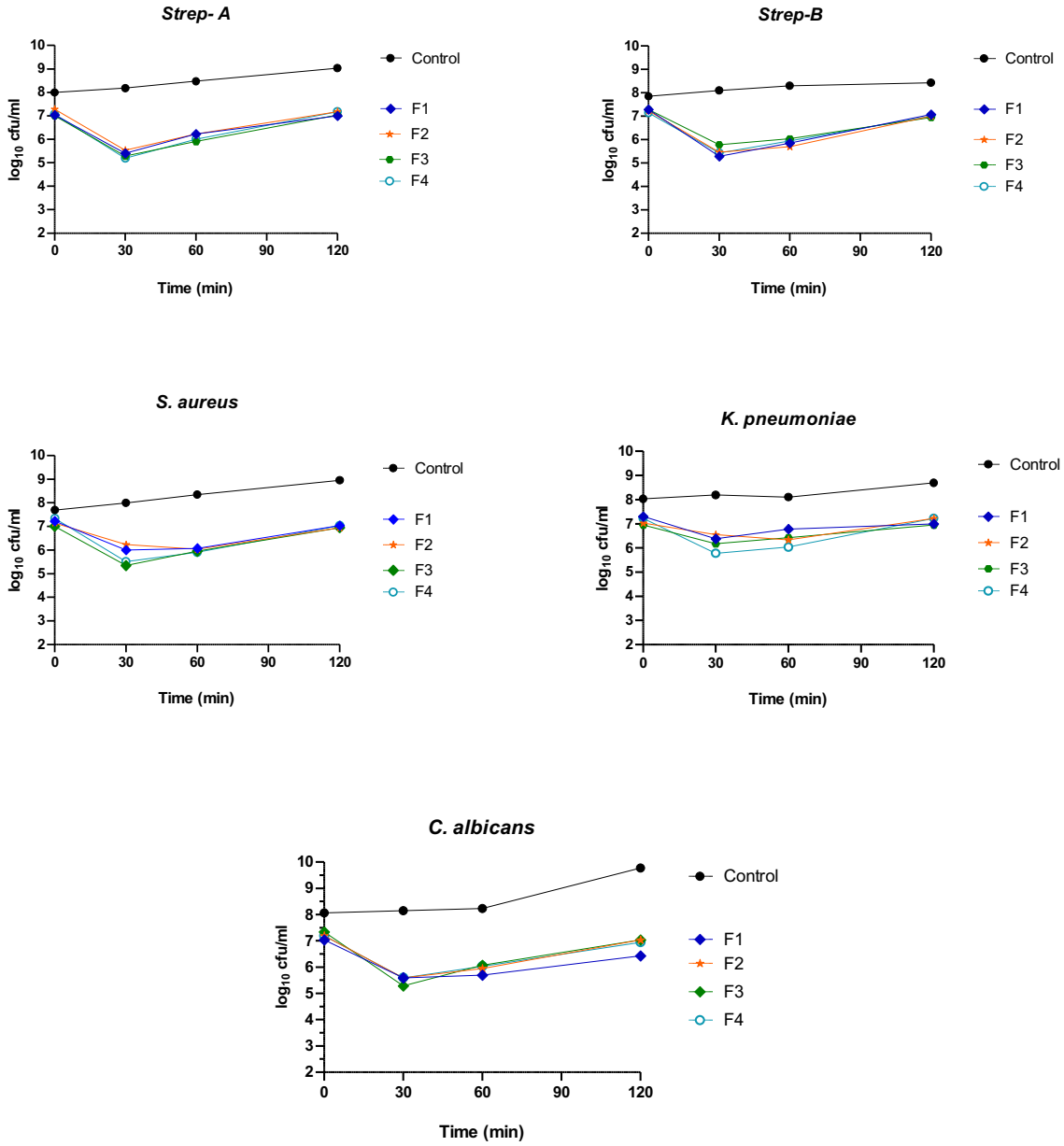

Figure 2. Time-kill determinations against *Strep-A*, *Strep-B*, *S. aureus*, *K. pneumoniae* and *C. albicans* strains after treatment with the lozenge formulations. The x-axis represents the killing time (min), and the y-axis represents the logarithmic bacteria/fungi survival.

4. MATERIALS AND METHODS

4.1. Materials

Honey, propolis (water-based), perga and oil of seaweed (*Chondrus crispus*), essential oils of pine turpentine (*Pinus spp.*), juniper (*Juniperus communis*), eucalyptus (*Eucalyptus globus*), sage (*Salvia officinalis*), lemon balm (*Melissa officinalis*) and tea tree (*Melaleuca Alternifolia*) were purchased from Arpaş Arifoğlu (Istanbul, Türkiye). Gum arabic, citric acid and powdered sugar were obtained from Sigma. All other chemicals were in pharmaceutical grade.

4.2. Preparation of lozenges

Firstly, the components of the formula are weighed and dissolved in citric acid and propolis (water-based) in a glass beaker, then honey and Perga are added and mixed until a homogeneous appearance is obtained. The weighed essential oils are added and dispersed in the aqueous phase. Weighed powdered sugar

and gum Arabic are mixed in a glass mortar, and the aqueous phase in the beaker is added in portions, and mixing is continued using a pestle until it becomes a homogeneous paste. The unit lozenges, which are filled and shaped into round moulds, are left to dry in an oven at 22°C and 60% relative humidity for 48 hours. The ingredients and their amounts of lozenge formulations were given in Table 4.

Table 4. Ingredients and their amounts of lozenge formulations.

Ingredients Percentage (%)	Formulation Number			
	F1	**F2**	**F3**	**F4**
Honey	7	7	7	7
Propolis (water based)	2	2	2	2
Perga	2	2	2	2
Powdered sugar	76	76	76	76
Gum arabic	9	9	9	9
Citric acid	0.5	0.5	0.5	0.5
Seaweed oil	1	1	1	1
Pine turpentine essential oil	1	1	1	1
Juniper essential oil	0.5	0.5	0.5	0.5
Eucalyptus essential oil	1	-	-	-
Sage essential oil	-	1	-	-
Lemon balm essential oil	-	-	1	-
Tea tree essential oil	-	-	-	1

4.3. Characterization of lozenges

4.3.1. Organoleptic properties

Organoleptic characterization of the prepared lozenges was carried out to evaluate their properties such as taste, odor, color, softness, surface smoothness, and shape [33].

4.3.2. Weight variation

20 lozenges per formulation were weighed individually on a precision balance (Mettler Toledo XPR404S, Mettler Toledo AG, USA) and the average weight of the lozenges was calculated. The average weight deviation values of the lozenge formulations were calculated as percentages (%). As per USP specification, the percentage deviation should not be more than 5% [25].

4.3.3. Diameter and thickness

The diameter and thickness of 20 lozenges per formulation were measured separately using a vernier caliper (Wert AG, China). The average diameter and thickness values in mm and standard deviation of the lozenge formulations were calculated [34].

4.3.4. Friability

10 lozenges per formulation were weighed together on precision balance and then, the pre-weighed lozenges were rotated for 4 minutes in the friabilator (Sotax FT2, Sotax AG, Switzerland) at 25 rpm. The tablets

were re-weighed and the friability values of the lozenge formulations were calculated as a percentage (%). As per USP specification, the percentage friability value of the lozenges should not be more than 1% [27].

4.3.5. In vitro disintegration time

In vitro disintegration time study was carried out according to the method described in USP, using a disintegrator (Sotax DT50, Sotax AG, Switzerland). Six lozenges per formulation were tested simultaneously through 10 mL of disintegration medium of phosphate buffer with pH 6.75 maintained at 37 ± 0.5°C and 100 rpm. 10 mL volume and pH 6.75 of the buffer were chosen because of mimicking the oral cavity medium after sucking the lozenges [35]. The study was terminated immediately when there was no residue at the bottom of the basket. The results were calculated as the mean value ± standard deviation [36]. Artificial saliva (at pH 6.75) was prepared according to the formulation given in Table 5 [37].

Table 5. Composition of the artificial saliva [37]

Ingredients	Quantity
Disodium hydrogen phosphate	2.382 g
Potassium dihydrogen phosphate	0.190 g
Sodium chloride	8.000 g
Ultrapure water	Up to 1 L
Phosphoric acid	q.s to pH 6.75

4.4. In vitro antimicrobial activity

4.4.1. Microorganisms

For antimicrobial activity experiments, the clinical isolate of group A, and group B beta-hemolytic *Streptococcus* (GABHS, GBBHS) from Clinical Microbiology Laboratories of Istanbul University, Istanbul Faculty of Medicine, and standard strains *Staphylococcus aureus* ATCC 29213, *Klebsiella pneumoniae* ATCC 4352 and, the yeast *Candida albicans* ATCC 10231 were used. Inoculums of bacteria and *C. albicans* were prepared with overnight cultures, for producing a concentration of $1x10^8$ colony-forming units (cfu/ml) and 1×10^7 cfu/ml, respectively.

4.4.2. Media

Cation-adjusted Mueller-Hinton broth (CAMHB, Difco Laboratories), and Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma) buffered to pH 7.0 with morpholine propane sulfonic acid (MOPS, Sigma) were used to determine the antibacterial and antifungal activities, respectively. Tryptic soy agar (TSA, Difco Laboratories), and Sabouroud dextrose agar (SDA, Difco Laboratories) were used for colony counts. Clinical isolates of GABHS and GBBHS were cultured in CAMHB or TSA supplemented with 5% sheep blood, under a 5% CO_2 atmosphere.

4.4.3. Determination of minimum inhibitor concentrations (MIC)

For determining the in vitro antibacterial and antifungal activities of the prepared lozenges, MICs were determined by the microbroth dilution technique as described by the Clinical and Laboratory Standards Institute [38,39]. Serial two-fold dilutions of lozenges which were disintegrated in a 5 ml artificial saliva (at pH 6.75) for 5-6 minutes, were prepared in CAMHB and 5% sheep blood supplemented CAMHB for bacteria, and RPMI-1640 medium for fungi in 96 well, U-shaped, polystyrene, sterile microtiter plates (Greiner). Since the lozenges will be diluted by 1/2 according to the test procedure, they were prepared in 2 times higher concentrations in 5 ml artificial saliva instead of 10 ml. Each well was inoculated with 50 µL of fresh broth cultures that gave a final concentration of 5×10^5 cfu/mL for bacteria, and 5×10^3 cfu/mL for fungi in the test tray. The trays were covered and placed in plastic bags to prevent evaporation, and the inoculated microplates were incubated for 18-24 hours at 37°C. The MIC values were defined as the lowest concentrations that produced complete inhibition of visible growth. Ciprofloxacin and fluconazole were used as reference

antibiotic and antifungal, for bacteria and fungi, respectively. The MIC values of the standard antibiotic and antifungal against standard strains were within the accuracy range according to CLSI [40].

4.4.4. Time kill curve (TKC) study

The TKC method described previously by the National Committee of Clinical Laboratory Standards [25] was used to determine the dynamic bactericidal and fungicidal activities of the prepared lozenges. For this purpose, 24-hour fresh broth cultures of microorganisms at a final concentration of $1x10^6$ cfu/ml, and the lozenges which were disintegrated in a 5 ml artificial saliva (at pH 6.75) for 5-6 minutes were mixed in equal volumes. Since the lozenges will be diluted by 1/2 according to the test procedure, they were prepared in 2 times higher concentrations in 5 ml artificial saliva instead of 10 ml. The tubes were incubated for 0, 30, 60, and 120 minutes at 37°C, samples were taken from each tube, serial 1/10-fold dilutions were made and 100 µL samples were plated on TSA or SDA. Colonies were counted 24 h after incubation at 37°C. An antimicrobial-free control of each strain was also included in the test.

TKCs were constructed by plotting mean colony counts (log10 cfu/mL) versus time. The lower limit of detection for the time-kill assays was 2 log10 cfu/mL. Antimicrobial carry-over was controlled by the inhibition of colonial growth at the site of the initial streak according to NCCLS guidelines. The bactericidal or fungicidal activity was defined as ≥3 log10 cfu/mL decrease from the initial inoculum [41].

Acknowledgements: Input text here.

Author contributions: Concept – İ.E.G.; Design – İ.E.G.; Supervision –İ.E.G., S.D.; Resources – İ.E.G., L.A., S.T., C.Y., F.N.Y., S.D.; Materials – İ.E.G.; Data Collection and/or Processing – İ.E.G., L.A., S.T., C.Y., F.N.Y., S.D.; Analysis and/or Interpretation – İ.E.G., L.A., S.T., C.Y., F.N.Y., S.D.; Literature Search – İ.E.G., L.A., S.T., C.Y., F.N.Y., S.D.; Writing – İ.E.G., C.Y., S.D.; Critical Reviews – İ.E.G., S.D.

Conflict of interest statement: The authors declared no conflict of interest.

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