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Development of herbal and vegan oral spray and its antimicrobial activities against pathogenic bacteria, fungi and viruses

Fatıma Nur Yılmaz [1], Dilek Matur [2], Bekir Özer [1,3] & Sibel Döşler [1]

[1] İstanbul University, Faculty of Pharmacy, Department of Pharmaceutical Microbiology, İstanbul, Türkiye
[2] Kurtsan Ilaçlari A.Ş., İstanbul, Türkiye
[3] İstanbul University, Institute of Graduate Studies in Health Sciences, İstanbul, Türkiye

Abstract

Background and Aims: Because the mouth and throat are the most important entrances of the body for several pathogens, using the nasal and oral antisepsis would be beneficial to fight with upper respiratory tract infections. In this study, a completely natural and vegan/herbal oral spray was developed and formulated, and its antimicrobial activities were tested against important mouth and upper respiratory tract pathogens.

Methods: The minimum inhibitory concentrations (MIC), time-kill curves (TKC), antiviral activities, and cytotoxic activities (against A549 and VERO cell lines), of the formulated oral spray, the active ingredient mixture, and/or the active substances individually, were determined against group-A beta haemolytic Streptococcus (GABHS), *Streptococcus pyogenes* ATCC 19615, *Staphylococcus aureus* ATCC 29213, *Klebsiella pneumoniae* ATCC 4352, the yeast *Candida albicans* ATCC 10231, and Parainfluenza virus type-2.

Results: The MIC values of the studied active herbal substances individually ranged from 0.4%-25%, while those for the spray formulation were 0.4-3%. TKC analysis showed that the oral spray has strong microbiocidal activities (≥3-Log_{10}) against all studied bacteria and yeast. Against parainfluenza virus type-2, the oral spray and the active ingredient mixture had strong antiviral activity (78% and 68%, respectively), without any cytotoxic effect.

Conclusion: According to our findings, the formulated and developed oral spray has several advantages such as having antiviral, antibacterial, and antifungal activities, being completely natural and vegan, free of chemical antiseptics, creating a moisturising and soothing protective layer with its barrier effect, and the ease of use of the spray form.

Keywords Oral spray · Development · Dormulation · Antibacterial · Antifungal · Activity

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Corresponding author: Sibel Döşler sibel.dosler@istanbul.edu.tr

INTRODUCTION

The first and most common symptoms of many diseases are burning and a dry feeling in the throat, a sore throat, felt as a scratching or irritation. These problems are usually caused by upper respiratory tract infections, such as common cold or influenza, and sometimes by dry or dusty air. Almost everyone suffers from a sore throat at least once in their lifetime, and certain factors increase that risk. Viruses are the most common causes of sore throat and the common cold, followed by bacteria and some fungi. In recent years, especially with the Coronavirus disease 2019 (Covid-19) pandemic, realisation and sensitivity to all kinds of upper respiratory tract infections has been increased. Therefore, it has become necessary to take additional measures to prevent all kinds of upper respiratory tract infections (Grief, 2013; Wang et al., 2021).

Since the mouth and throat are the most important entrances to the body, for respiratory tract and also all kinds of infections, there is an inverse proportion between the use of several antimicrobial oral products and the risk of such infections. Several studies are shown that these products slow down the disease progression and prophylaxis by reducing the viral load, especially in the early periods of infections. Thus, the use of nasal and oral antisepsis, as well as personal protective equipment, has been reported as beneficial to fight respiratory tract infections. In this context, besides the drugs containing chemical active substances and/or chemical preservatives that can be used for treating or alleviating the symptoms of sore throats, various herbal medications such as oral sprays, mouthwashes, drops, or pastilles can also be utilised (Mukherjee et al., 2017; Golac-Guzina et al., 2019; Seet et al., 2021; Gudmundsdottir; 2021). Most of the existing herbal oral sprays contain chemical additives, dyes, animal origin substances, and synthetic preservatives etc. Although, most of these products are currently in widespread use, and the safety concerns thought as minimum, their possible negative effects on health such as alteration of oral flora, staining of teeth, irritation of epithelium, development of cross-resistance with antibiotics, especially in their long-term use are still remaining (Stathis et al., 2021).

To eliminate these disadvantages, there is a need to create an effective and safe alternative product containing natural/herbal active ingredients. For this purpose, a new oral spray with entirely herbal, natural, and vegan properties was developed in this study. It contains active compounds that have been safely used by the public and in alternative or supportive medicine for many years. It was also aimed to determine the antimicrobial activities of the developed oral spray against various bacteria, fungi, and virus that can cause infections in the mouth, throat, and especially in the respiratory tract.

MATERIALS AND METHODS

Microorganisms

The clinical isolate of group A beta haemolytic Streptococcus (GABHS) from the Clinical Microbiology Laboratories of Istanbul University, Istanbul Faculty of Medicine, and standard strains of *Streptococcus pyogenes* ATCC 19615, *Staphylococcus aureus* ATCC 29213, *Klebsiella pneumoniae* ATCC 4352, the yeast *Candida albicans* ATCC 10231, and Parainfluenza virus type-2 (PIV-2) were used. Inoculum of bacteria and *C. albicans* were prepared with overnight cultures to produce a concentration of $1x10^8$ colony-forming units (cfu/mL) and $1x10^7$ cfu/ml, respectively.

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 Sabouraud dextrose agar (SDA, Difco Laboratories) were used for colony counts. *S. pyogenes* ATCC 19615 and a clinical isolate of GABHS were cultured in CAMHB or TSA supplemented with 5% sheep blood, under a 5% CO_2 atmosphere.

For PIV-2, the African green monkey kidney cell line (VERO, ATCC) was cultured in Dulbecco's modified Eagle's medium (Wisent, MULTICELL) supplemented with 6% fetal bovine serum (FBS; Sigma), 100 U/mL penicillin, and 100 µg/mL streptomycin. The PIV-2 cells were maintained at 37°C under a 5% CO_2 atmosphere.

Development of the Formulation

The formulation contains tea tree oil from *Melaleuca alternifolia* (Maiden & Betche) Cheel, peppermint oil from *Mentha piperita* L, clove oil from *Eugenia caryophyllata* Thunb., eucalyptus oil from *Eucalyptus globulus* Labill., and thyme extract from *Thymus vulgaris* L.. Based on the literature search, these active substances have been carefully selected according to their desired activities and suitability for effectiveness and taste.

While the selection of excipients, the substances meeting the purposes such as helping the solubility of active ingredients, providing the expected taste, preventing the microbiological contamination, and facilitating the application were preferred. For these purposes, we used glycerine, saccharide, polysorbate-80, *Salvia officinalis* L. hydrosol, and deionised water in the formulation.

Stability of the Formulation

Stability tests were carried out under two different conditions: short-term (accelerated) and long-term. Short-term conditions: Temperature 40°C±2°C, relative humidity 75%±5%, and duration 6 months; Long-term conditions: Temperature 25°C±2°C, relative humidity 60%±5%, and the duration as 24 months, which is the expected shelf life. Under these conditions, tests on the physicochemical properties and antimicrobial activities of the product were repeated to ensure that there were no negative changes compared to the initial state.

Determination of the Minimum Inhibitor Concentrations (MIC)

For determining the in vitro antibacterial and antifungal activities of the formulated oral spray; the mixture of active substances (powdered thyme extract, peppermint oil, tea tree oil, eucalyptus oil, and clove oil); and each substance separately, MICs were determined by the microbroth dilution technique as described by the Clinical and Laboratory Standards Institute (CLSI, 2000; CLSI, 2006). Serial two-fold dilutions of antimicrobials were prepared in CAMHB or 5% sheep blood supplemented with CAMHB for bacteria, and RPMI-1640 medium for fungi in 96-well microtiter plates. 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 incubated for 18-24 h 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 the reference antibiotic and antifungal, respectively.

Time-Killing Curve (TKC) Studies

The TKC method was used to determine the dynamic bactericidal and fungicidal activities of the oral spray, the mixture of active ingredients, and each active ingredient alone as previously described by the National Committee of Clinical Laboratory Standards (NCCLS, 1999). For this purpose, 24 h fresh broth cultures of microorganisms at a final concentration of $1x10^6$ cfu/mL and the antimicrobial substances were mixed in equal volumes. Because the product or its components were diluted 1/2 as per during the procedure, all antimicrobial substances were prepared at 2x concentrations. The tubes were incubated for 0, 2, 4, 6, or 24 h at 37°C, samples were taken from each tube, serial 1/10-fold dilutions were made, and 100 µL samples were plated on the appropriate medium. Colonies were counted 24 h after incubation at 37°C. An antimicrobial-free control of each strain was also included to the tests.

TKCs were constructed by plotting the mean colony counts ($\log_{10}$ cfu/mL) versus time. The lower limit of detection was 1-$\log_{10}$ cfu/mL. Also, antimicrobial carry-over test was performed by inhibiting colonial growth at the site of the initial streak according to the NCCLS guidelines (NCCLS, 1999). Bactericidal or fungicidal activity was defined as a ≥3-$\log_{10}$ cfu/mL decrease from the initial inoculum.

Antiviral Activity Assay

An experiment was conducted based on the plaque formation test for the PIV-2 strain grown on VERO cell culture (Fukushima et al., 2022). VERO cells were infected with PIV-2 at 100 PFU per well, one of the wells was used as a control, and the antiviral activities of the oral spray, mixture of active ingredients, and each active ingredients were tested in the other wells. The virus-infected control well was accepted as 100%, and the efficacy results were calculated as a percentage by comparing the control.

Cytotoxicity Assay

Oral spray was tested against the A549 and VERO cell lines (Oyardi et al., 2022; Yildiz et al., 2022). Cells were grown in Eagle's Minimum Essential Medium (EMEM) (Gibco) supplemented with 10% FBS (Gibco) and 100 units per mL penicillin G and streptomycin under a humidified, 5% CO_2 atmosphere at 37°C. The cells were seeded as 1×10^4 per well in 96-well plates and allowed 24 h to ensure the attachment, then they were incubated in the absence or presence of oral spray (serial dilutions between 8 times above and 4 times below) for 24 h. To test the concentrations above the normal usage dose, all antimicrobial substances were prepared at 8x concentrations.

Cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay in accordance with the manufacturer's protocol. After incubation, the MTT solution was removed from the wells, DMSO solution (Sigma, 100 µL) was added, and the plates were placed on a shaker for 15 min. The optical densities were measured at 570 nm (EON-BioTek Instruments, Winooski, VT, USA). Three replicates were used for each concentration, and the process was repeated three times. The cytotoxic effects of each compound were obtained as 50% inhibitory concentrations (IC_{50}) values, which represent the molar drug concentrations required for 50% inhibition of cell viability, calculated by GraphPad Prism version 8.

RESULTS

Manufacturing Process

The amounts of active substances and excipients in the formula were determined by considering the literature data,

preliminary tests, and experimental feedback since the sensory taste is important as well as the efficacy. Great care has been taken to ensure that all the selected excipients are available in the required minimum number and diversity, and comply with the relevant legal regulations.

The main carrier and solvent of the product is deionised water. In the production stages, the excipients used to regulate the taste were dissolved in water, then standardised powdered thyme extract dispersed homogeneously in water was added and mixed. The essential oils were mixed with the emulsifier polysorbate-80 to obtain a stable water-based emulsion and added to the mixture. Finally, the formulation was mixed to be completely homogeneous and filtered.

Stability of the Formulation

Because of the stability tests conducted under two different conditions, it was determined that the product remained physicochemically stable and maintained its microbiological activity under all test conditions.

Minimum Inhibitory Concentrations (MICs)

The in vitro antimicrobial activities of the oral spray, the mixture of active ingredients, and each active ingredient are summarised in Table 1. The MIC values of the standard antibiotic and antifungal against the standard strains were within the accuracy quality control ranges according to CLSI (2022).

Time-Killing Curve (TKC) Analyses

As shown in Figure 1, developed oral spray was found to be much more effective against all tested infectious agents compared to the controls or individual components in the product. That activity against almost all studied bacteria and yeast, started at 2th h, and reached the highest level at 4th h by showing a strong microbiocidal rate as ≥3-log$_{10}$. When the active ingredients were tested as a mixture, similar to the spray, they found more effective than the controls or individuals, but the individuals alone, showed only static activities in the first 4-6 h, and regrowth was observed at 24th h.

Antiviral Activities

As shown in Figure 2, both the formulated oral spray and the active ingredient mixture were effective directly (78% and 68%, respectively) and were also diluted as ½ times (39% and 34%, respectively) against the PIV-2. While these effects were compared with each other, the spray form was at least 10% more active than the mixture, and the individuals' activities were between 38% and 46% (Table 2).

Table 2. Antiviral activities of the oral spray and the active substances (%).

Substance	Viral inhibition
Oral spray (direct)	78
Oral spray (1/2 dilution)	49
Mixture of the active ingredients (direct)	68
Mixture of the active ingredients (1/2 dilution)	34
Powdered thyme extract	40
Peppermint oil	46
Eucalyptus oil	38
Clove oil	41
Tea tree oil	39

Cytotoxicity Assay

According to the cytotoxicity assays, the IC$_{50}$ values of the oral spray for the A549 and VERO cell lines were found to be 3.8 and 3.6 times greater than its developed concentration, respectively. These results showed that the oral spray does not have any cytotoxic side effect at the usage concentration.

Table 1. Minimum inhibitor concentrations of the active substances (%).

	S. pyogenes ATCC 19615	AGBHS, Clinical isolate	*S. aureus* ATCC 29213	*K. pneumoniae* ATCC 4352	*C. albicans* ATCC 10231
Oral spray	0.8	0.4	3	1.5	1.5
Mixture of the active ingredients	1.5	1.5	3	3	3
Powdered thyme extract	25	25	25	6	25
Peppermint oil	0.8	1.5	1.5	3	3
Eucalyptus oil	1.5	6	3	0.4	0.8
Clove oil	0.4	1.5	6	25	6
Tea tree oil	0.8	1.5	1.5	3	3

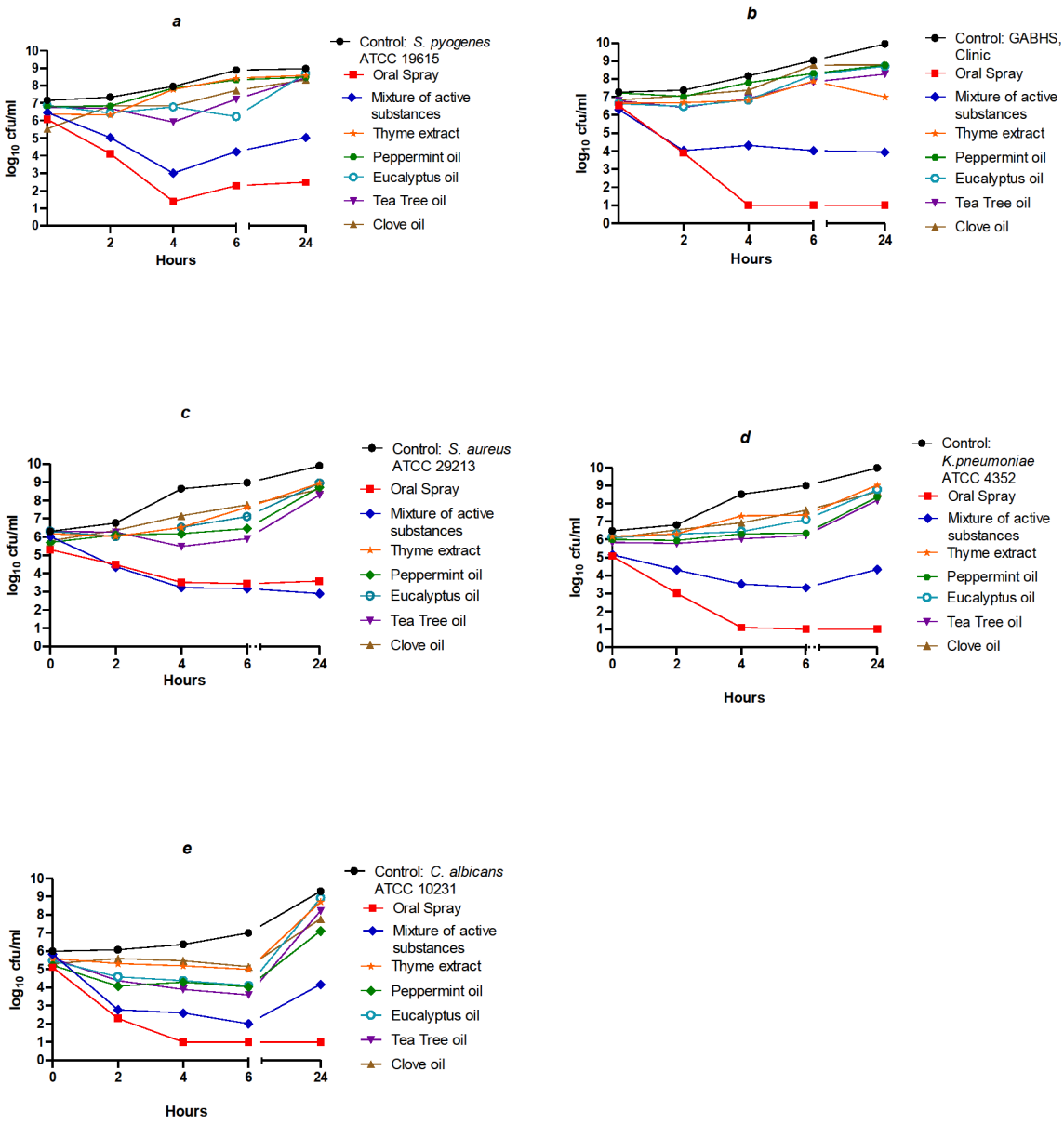

Figure 1. Time killing curves of oral spray and its active substances as a mixture or alone against a: standard *S. pygenes*, b: clinical strain of *S. pygenes* (GABHS) and standard strains of c: *S. aureus*, d: *K. pneumonia*, and e: yeast *C. albicans*. X-axis represents time as hours, Y-axis represents the number of survival microorganisms (log cfu/mL). Control: Bacterial / fungal suspension not treated with any antimicrobial agent.

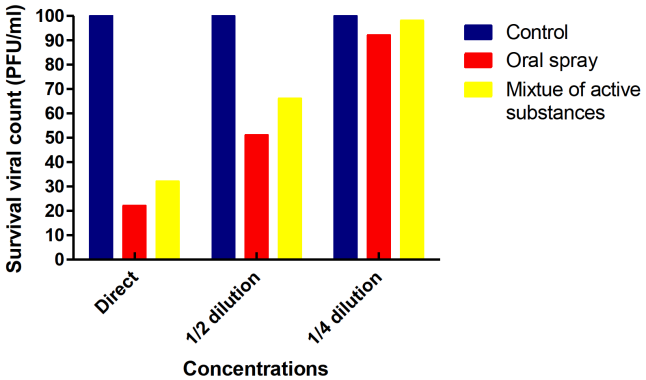

Figure 2. Antiviral activities of oral spray and mixture of active substances at direct, 1/2 and ¼ dilutions comparing with the control. X-axis represents concentrations, Y-axis represents the number of survival viral counts (pfu/mL). Control: Viral suspension not treated with any antimicrobial agent.

DISCUSSION

Recently, it has become very important to find safe and effective alternative control strategies or treatments for infectious diseases in parallel with the increasing antimicrobial-resistant microorganisms and the inadequacy of current new antibiotics, antifungal or antiviral drugs. One of the novel strategies involves using herbal agents because the plants have been the main source for pain relief or the treatment of various diseases since ancient times. As mentioned by Pan et al. (2013), there are lots of evidences for using the plants in treatment dates back to the BC ages, and most of the existing drugs are plant-based.

All over the world, especially with the coronavirus pandemic, there are increasing numbers of products on the market for protecting from respiratory tract infections. Among them, various over-the-counter oral sprays are available and are generally approved for application to the oral mucosa to relieve the symptoms. They are also used for reducing the duration of disease and increasing the viral or bacterial clearance of the upper respiratory tract. In particular, natural and/or herbal formulations are widely preferred for their safety of use and health-promoting properties (Olczyk et al., 2017; Xiong et al., 2020; Schütz et al., 2021; Khorshiddoust et al., 2022).

In this study, we designed an herbal and natural oral spray that can help prevent and/or treat all kinds of infections in the mouth, throat, and especially the upper respiratory tract. Also, we targeted the soothing effect for patients, and easy to use formulation with its pleasant taste and smell. Since several plants have synergistic effects with each other, we developed a combined ingredient by making a meticulously and carefully literature review, and pilot experiments for each active substance.

Among the active ingredients, the tea tree (melaleuca) oil from *M. alternifolia* has become increasingly popular in recent decades as a complementary and alternative medicine. Tea tree oil, which comprises terpene hydrocarbons and their associated alcohols, has been used for almost 100 years in Australia and is now available worldwide with its antiseptic and anti-inflammatory activities (Carsonet al., 2006; Sharifi-Rad et al., 2017). Eucalyptus oil from *E. globulus*, and its major component 1,8-cineole, have strong antimicrobial effects against various bacteria, including *Mycobacterium tuberculosis* and methicillin-resistant *S. aureus* (MRSA), viruses, and fungi; they also have immune-stimulatory, anti-inflammatory, antioxidant, analgesic, and spasmolytic effects (Sadlon & Lamson, 2010).

Clove oil from *E. caryophyllata*, which contains eugenol (70–95%), eugenol acetate (up to 20%) and β-caryophyllene (12–17%), is a colourless or yellow liquid, obtained from the distillation of clove buds. *E. caryophyllata*, also known as *Syzygium aromaticum* (clove), is one of the most valuable and second most crucial spice crops in the world trade, and The United States Food and Drug Administration listed clove oil as "Generally Regarded as Safe" for humans. Since ancient times, cloves have been used with its various biological properties such as anti-inflammatory, anaesthetic, antimicrobial, antifungal, antiviral, leishmaniacidal, antioxidant, anticancer, nematocidal, herbicidal, acaricidal, and larvicidal (Xu et al., 2016; Batiha et al., 2020).

Peppermint oil from the aerial parts of *M. piperita*, comprising menthol (36.02%), menthone (24.56%), menthyl acetate (8.95%), and menthofuran (6.88%), as the major components. It has been used for centuries in the community and shows significant antibacterial and antifungal activity against various pathogens (Camele et al., 2021; Silva et al., 2022). The last active ingredient, thyme from *T. vulgaris*, is considered an important wild edible plant, studied for centuries for its unique importance in the food, pharmaceutical, and cosmetic industry. Thyme contains phytonutrients, minerals and vitamins and is mainly composed of flavonoids and antioxidants, especially thymol and carvacrol. Besides its multi-pharmacological effects such as antioxidant, anti-inflammatory, and antineoplastic actions, thyme has long been known for its antiviral, antibacterial, antifungal, and antiseptic activities (Halat et al., 2022).

The antimicrobial activities of the mentioned active ingredients were tested in this study. According to the results, all of them showed antimicrobial activity at a concentrations ranging from 0.04% to 25%, depending on the microorganism (Table 1). In the oral spray formulation, due to their synergistic interactions, these substances have been used as 2-2500 times lower than their effective concentrations. When we determined the MICs of the active ingredient mixture and the whole formulation against the same bacteria and yeast, we observed that the MIC values of the mixture were between 1.5-3%, while those for formulation were 0.4-3%.

When we analysed the dynamic picture of the antimicrobial activities of the oral spray, the antibacterial and antifungal activities were started immediately, and at the 2nd h, the oral spray showed 2-3-log$_{10}$ -cidal effect against all studied microorganisms except *S. aureus*. Against *S. aureus,* activities were started at 4th h. On the other hand, when we tested the active substances alone, they generally showed a -static effect during the first 6 h, but the regrowth was observed at 24th h. The mixture of active ingredients also showed greater antimicrobial activity, as expected, but it was lower than that of the oral spray. Especially at 24th h, the spray form showed at least

2-$\log_{10}$ lethal effect against the studied microorganisms. It is thought that these differences are provided by the positive interactions with other excipients in the formulation, besides some other positive interactions such as ensuring long-term stability, preventing chemical incompatibilities, and providing lyphophilic/hydrophilic balance.

Most of the upper respiratory tract infections are caused by viruses, instead of bacteria, and this makes it essential to know the antiviral activities of products applied to the mouth or throat. Human parainfluenza viruses are single-stranded, enveloped RNA viruses from the *Paramyxoviridae* family, which are important respiratory pathogens for children and adults, causing a wide range of infections such as colds, croup, bronchiolitis, and pneumonia (Branche & Falsey, 2016). When we tested the oral spray formulation against the PIV-2, we observed very good antiviral activity, such as 78%, and this activity continued at a rate of 49% even when the spray was diluted in half. Similarly, the mixture of active ingredients showed good antiviral activity as 68%, and continued as 34% at ½ dilution. On the other hand, just as the antibacterial and antifungal activities, the active ingredients had lower antiviral activities such as 38%-46%.

The use of antiviral agents that reduce the viral load in the upper respiratory tract could help prevent the infections and reduce the virus spreading among people. Some of the commercially available oral or nasal sprays show good virucidal activity at high concentrations, but they also produce cytotoxic side effects against mucosal cells (Kramer et al., 2021). In this study, although the formulated oral spray contained only natural and herbal ingredients that have been safely used in society for decades at very low concentrations, it was tested on A549 and VERO cell lines to determine any potential cytotoxic effects if used at high doses through repeated spraying. According to our cytotoxicity test results, the IC_{50} dose of the formulation is approximately four times higher than the usage concentration, indicating that the product can be safely used without any potential to cause toxic side effects.

CONCLUSION

The formulated natural and vegan oral spray has high antimicrobial activities in a short time against upper respiratory tract and throat pathogens such as the common infectious virus PIV-2, the most common pathogenic bacteria, and infectious yeast for the mouth. Compared with the other oral antimicrobial products, this formulation has several advantages such as being natural and vegan, having antiviral activity, not having cytotoxicity, being free of chemicals, creating a posturing and soothing protective layer, and the ease of use of the spray form. Thus, it could be a good candidate as an alternative to commercially available oral products.

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Author Details

Fatıma Nur Yılmaz
[1] İstanbul University, Faculty of Pharmacy, Department of Pharmaceutical Microbiology, İstanbul, Türkiye
0000-0001-8442-8538

Dilek Matur
[2] Kurtsan Ilaçlari A.Ş., İstanbul, Türkiye
0009-0000-5734-6262

Bekir Özer
[1] İstanbul University, Faculty of Pharmacy, Department of Pharmaceutical Microbiology, İstanbul, Türkiye
[3] İstanbul University, Institute of Graduate Studies in Health Sciences, İstanbul, Türkiye
0000-0002-3482-5081

Sibel Döşler
[1] İstanbul University, Faculty of Pharmacy, Department of Pharmaceutical Microbiology, İstanbul, Türkiye
0000-0001-5223-4755 sibel.dosler@istanbul.edu.tr

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