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Development of propolis and essential oils containing oral/throat spray formulation against SARS-CoV-2 infection

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ABSTRACT

A broad range of evidence has confirmed that natural products and essential oils might have the potential to suppress COVID-19 infection. Therefore, this study aimed to develop an oral/throat spray formulation for prophylactic use in the oral cavity or help treatment modalities. Based on a reference survey, several essential oils, a cold-pressed oil, and propolis were selected, and cytotoxicity and antiviral activity of each component and the developed spray formulation were examined against severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection using Vero E6 cells. Anti-inflammatory, antimicrobial, and analgesic activities as well as mutagenicity and anti-mutagenicity of the formulation were analysed. Forty-three phenolics were identified in both propolis extract and oral/throat spray. The spray with 1:640-fold dilution provided the highest efficacy and the cytopathic effect was delayed for 54 h at this dilution, and the antiviral activity rate was 85.3%. A combination of natural products with essential oils at the right concentrations can be used as a supplement for the prevention of SARS-CoV-2 infection.

1. Introduction

Humans have suffered from numerous epidemics and pandemics that have affected hundreds of millions of lives throughout the history. Just a century after the last disaster of Spanish pandemia in 1918, severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection started to affect people globally and caused severe and fatal illnesses in humans. Despite such significant advances in contemporary medicine, the virus has continued to challenge not only human lives but has also threatened the global economic security and healthcare systems. The scientific community has been burdened with developing effective prophylactic and treatment options intensively. Consequently, several types of vaccines and drugs have been developed and offered clinical relevance.

A reference survey was carried out in PubMed with the keyword "corona pandemic" and 127,000 published manuscripts were found since the pandemic in 2020. However, only a small fraction of them (803) (as of Feb 2022) were related to herbals, and these mainly reviewed the possible herbal candidates hypothetically or from the results of *in silico* molecular docking studies. Actually, the number of experimental evidence published on the antiviral effects of herbals against viral infections is limited due to the required laboratory conditions. On the other hand, treatment approaches with common antiviral chemotherapeutics have remained inconclusive against SARS-CoV-2 infection. Therefore, it is obvious that the effect of a drug or herbal remedy candidate should be tested against the specific SARS-CoV-2 strains.

Since the nasal and the oral cavity have been reported as the main contact area with the body, this study aimed to develop an oral/throat

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spray formulation for prophylactic use in the oral cavity or to help with treatment modalities. For the selection of herbal components to be included in the formulation, we reviewed the reported scientific evidence published thus far with the keywords "covid, essential oil, and immunity" (Banerjee et al., 2021; Javed, Meeran, Jha, & Ojha, 2021; Valussi, Antonelli, Donelli, & Firenzuoli, 2021; Wani, Yadav, Khursheed, & Rather, 2021; Yosri et al., 2021). Based on *in vitro* and *in silico* evidence, essential oils from oregano (carvacrol), trilobed sage (1,8-cineol), peppermint (menthol), lemon peel (citral), and geranium (geraniol) as well as cold-pressed black cumin seed oil (nigelline and thymoquinone) and black poplar propolis (caffeic acid phenethyl ester; CAPE) were selected as the main active components of the formulation. The ratio of these components in the formulation was adjusted based on the experimental antiviral and antimicrobial tests and provided a tolerable taste in the mouth. Besides the formulation's efficacy, the safety profile of this formulation was studied in detail.

2. Materials and methods

2.1. Materials

Propolis extract with minimum 0.65% CAPE content, cold-pressed black cumin (*Nigella sativa* L.) oil, Anatolian sage (*Salvia triloba* L.) essential oil (minimum 20% 1,8-cineol), oregano (*Origanum onites* L.) essential oil (minimum 70% carvacrol), peppermint (*Mentha piperita* L.) essential oil, lemon peel (*Citrus limon*) essential oil, geranium (*Pelargonium graveolens* L.) essential oil, α-tocopherol, food-grade alcohol (96%), and medium-chain triacylglycerols (MCT) with 60% caprylic acid and 40% capric acid content were procured from Altıparmak Gıda San. & Tic. A.Ş., Istanbul, Turkey.

2.2. Reagents and standards

The compounds Na_2CO_3 (ACS grade, anhydrous, ≥ 99.5%), NaCl (ACS grade, ≥ 99.0%), and the analytical standards of phenolic acids and flavonoids [namely cinnamyl aldehyde, phloroglucinol, 3,4-dimethoxybenzaldehyde, 4-hydroxybenzoic acid, genistein, protocatechuic acid, *p*-coumaric acid, *trans*-cinnamic acid, 2-OH-coumaric acid, phenyl lactic acid, vanillic acid, homogentisic acid, gallic acid, shikimic acid, caffeic acid, *m*-coumaric acid, quinic acid, *trans*-ferulic acid, syringic acid, resveratrol, chrysin, pinocembrin, 3,4-dimethoxy cinnamic acid, apigenin, galangin, pinobanksin, methyl syringate, CAPE, kaempferol, luteolin, naringenin, (-)-epi-catechin, (+)-catechin hydrate, rutin hydrate, quercetin, (+/-)-taxifolin hydrate, epigallocatechin, isorhamnetin, hesperetin, ellagic acid, chlorogenic acid, myricetin, and rosmarinic acid] were purchased from Sigma-Aldrich® (Merck KGaA, Darmstadt, Germany). The purity of all analytical standards was above 98.8%. Acetic acid (ACS grade, ≥ 99.7%), ethanol (EtOH, absolute, ≥ 99.8%), Folin-Ciocalteu's phenol reagent, ethyl acetate (anhydrous, 99.8%), chloroform (HPLC grade, ≥ 99.8%), hexane (HPLC grade, ≥ 95%), formic acid (reagent grade, ≥ 95%), methanol (MeOH, LC grade), sodium nitrite, and acetonitrile (ACN, LC grade) were purchased from VWR International Laboratuvar Teknolojileri Ltd. Şti. (Istanbul, Turkey), unless otherwise stated.

For mutagenicity/anti-mutagenicity assays, *Salmonella typhimurium* bacterial strains and the metabolic activation system (S9) prepared from rat liver were supplied from Moltox Molecular Toxicology, Inc (Boone, NC, USA). 4-Nitro-o-phenylenediamine (NPD), sodium azide, and 2-aminofluorene were from Sigma-Aldrich® (Merck KGaA). The nutrient broth was obtained from HiMedia Laboratories Ltd. (Mumbai, Maharashtra, India).

Antimicrobial activity of samples was evaluated against six common oral/throat pathogens: gram-positive bacteria (*Staphylococcus aureus*-MRSA ATCC 25923, *Streptococcus pyogenes* ATCC 19615, and *Staphylococcus aureus*-MSSA), gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27353 and *Klebsiella pneumonia*), and yeast such as fungus (*Candida albicans* ATCC 90028). *Staphylococcus aureus*-MSSA and *Klebsiella pneumonia* clinical isolates were obtained from Istanbul University Hospital (Istanbul, Turkey). All strains were maintained at −80 °C in a vial containing cryoprotective fluid until used. In addition, brain heart infusion agar (BHIA; Merck KGaA), 5% sheep blood agar (SBA; GBL, Istanbul, Turkey), tryptone soy agar (TSA; Oxoid, Basingstoke, UK), and chlorhexidine gluconate 0.2% (w/v) (Klorhex-DentaSave, Ankara, Turkey) were obtained from the specified providers. The B.1.36 strain, utilized in SARS-CoV-2 antiviral efficacy tests, was generously provided by the Turkish Directorate of Public Health (Ministry of Health, Ankara, Turkey).

Vero E6 cells (ATCC®, CRL-1586TM, Manassas, VA, USA) were cultured in complete media consisting of Dulbecco's Modified Eagle's Medium (DMEM; HyClone, Cat # SH30021.01, Gibco, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone, Cat # SV30160.03, Gibco), 100 units/mL penicillin, and 100 g/mL streptomycin. Mycoplasma testing was performed at intervals on the cell lines using a MycoAlert PLUS Mycoplasma Detection Kit (Lonza, Cat # LT07-710, Basel, Switzerland).

The RAW264.7 murine macrophage cell line and L929 healthy mouse fibroblast was provided by American Type Culture Collection (Manassas, VA, USA) and was cultured in DMEM supplemented with 10% FBS, 1% penicillin (10.000 units/mL), and streptomycin (10.000 μg/mL) (Gibco) at 37 °C under a humidified atmosphere of 5% CO_2. Indomethacin, lipopolysaccharide (LPS) from *Escherichia coli* 0111: B4, and Griess reagent (1% sulfanilamide and 0.1 % N-(1-naphthyl)ethylenediamine dihydrochloride in 5% phosphoric acid) were purchased from Sigma-Aldrich® (Merck KGaA).

2.3. Development of oral/throat spray

Frozen crude propolis (−18 °C) was first grounded using a grinder and then extracted with 70% ethanol. Having stirred for 24 h at room temperature, the extract was kept at −18 °C for another 24 h and finally filtered to remove wax. For oral/throat spray formulation, the obtained propolis extract (in solid form) was ground with liquid nitrogen to fine particles and dissolved in food-grade alcohol in an ultrasound water bath (Bandelin, Berlin, Germany). Then, MCT was added to propolis solution in alcohol and vortexed for 2 min. After that, α-tocopherol was dissolved in cold-pressed black cumin oil. Finally, cold-pressed black cumin oil together with α-tocopherol and all essential oils (Anatolian sage, thyme, medical mint, lemon, and geranium) were combined with the alcoholic mixture of propolis and MCT by vortexing for 5 min. Table 1 shows the content and specification of oral/throat spray formulation. Moreover, preliminary molecular docking works of literature was used in the present study to develop the current formulation (Senthil Kumar et al., 2020).

2.4. Identification and quantification of phenolic profiles

2.4.1. Sample preparation

At the sample preparation step of phenolic profiling analysis, an in-house simultaneous extraction and clean-up method was developed. According to this protocol, a 0.2 mL of oral/throat spray was first taken into a 15 mL centrifuge tube. Then, 800 μL distilled water, 3.2 mL of EtOH, and 8 mL of hexane were added to the sample. The resulting heterogeneous solution was shaken using a rotary orbital shaker (MaxQ 4000 Benchtop Orbital Shaker, Thermo Fisher ScientificTM, Inc., Waltham, MA, USA) for 30 min to extract phenolics and for defatting the sample. After that, it was centrifugated at 3000 × *g* for 5 min, and the resulting lower phase was filtered to another tube using a 0.45 μm PVDF filter (Interlab®, Istanbul, Turkey). Then, a 25 μL aliquot of this solution was mixed with 500 μL water and 475 μL MeOH in an amber glass vial, and 10 μL of the sample was finally injected into Ultra Performance Liquid Chromatography-Tandem Mass Spectrometer (UPLC-MS/MS) (Waters®, Milford, MA, USA). For propolis extract, a 0.5 g of sample (in

Table 1
Content and specification of oral/throat spray formulation.

Content	Specification	Recommended Daily Oral Spray[a]	
		4 sprays/ day (mg)	6 sprays/ day (mg)
Propolis	min. 0.65% CAPE	10.80	16.20
Ethanol	96% food grade	[b]	[b]
MCT	60% caprylic acid and 40% capric acid	[c]	[c]
Cold-pressed black cumin oil	1.14% - thymoquinone	165.6	248.4
Anatolian sage essential oil	min. 20% 1,8-cineol	8.28	12.42
Oregano essential oil	min. 70% carvacrol	1.80	2.70
Lemon essential oil	min. 45% limonene	12.60	18.90
Geranium essential oil	min. 20% citronellol	[d]	[d]
Peppermint essential oil	Flavour enhancer	5.04	7.56
Alpha-tocopherol	Antioxidant against lipid peroxidation	[e]	[e]

Abbreviations: CAPE, caffeic acid phenethyl ester; MCT, medium chain triacylglycerols.
Notes: [a] Recommended daily amount is 4 to 6 sprays per day for adults and children (11 years of age and older). It is recommended to use 2 or 3 times a day, in the form of 2 sprays (Commercial Name: Apitera[Plus] Propolis Spray). [b] Used as solvent. [c] Used as emulsifier. [d] Used as natural flavour agent. [e] Used as antioxidant.

fine particles) was diluted with 70% ethanol (1:1000-fold) and thoroughly shaken. Then, a 100 μL aliquot of this solution was mixed with 900 μL of methanol:water (1:1) in an amber glass vial. Finally, a 10 μL of the sample was injected into UPLC-MS/MS system.

Stock standards of phenolics were prepared by dissolving each in ethanol individually at 2 mg/mL concentration. The phenolic stock standard mixture was prepared at a concentration of 0.01 mg/mL by adding 50 μL from each stock standard in a 10 mL volumetric flask and diluted to an appropriate volume. External calibration plots for each substance were generated at 6 linear calibration points (0.05, 0.1, 0.25, 0.5, 1.0, and 2.0 μg/mL) by serial dilution of stock standard mixture using MeOH/distilled water (50:50, v/v) solution. Phenolics were quantified based on their peak areas and comparison with a calibration curve obtained with corresponding standards.

2.4.2. Instrumentation and MS data acquisition parameters

Phenolic profiles were determined according to the UPLC-MS/MS method of Escriche and Juan-Borrás (2018), with slight modification. The equipment consisted of a Waters® ACQUITY UPLC binary solvent delivery system, a column oven, and an autosampler equipped with Waters® Xevo triple-quadrupole (TQ) MS/MS (Waters®). The analytical separation and chromatographic resolution were performed on Waters® CORTECS T3 column (1.6 μm, 2.1 × 150 mm) (Waters®) using a gradient elution of (A) water containing 0.01% acetic acid and (B) ACN/MeOH (80:20, v/v) containing 0.01% acetic acid at a flow rate of 0.25 mL/min. Run time was 40 min and applied gradient elution program was used as follows: 2% B (0–1.30 min), 2–55% B (1.30–35 min), 55–95% B (35–37 min), 95–2% B (37–37.01 min), and 2% B (37.01–40 min). The autosampler tray and column temperature were set at 10 and 30 °C, respectively.

The ion source and desolvation temperature were optimised for the best response and held at 150 and 450 °C, respectively, for MS/MS. Desolvation and cone gas flow rate was maintained at 850 and 50 L/h, respectively. 2 kV of needle capillary voltage provided the best ionisation efficiency. Multiple Reaction Monitoring mode was employed and the peak areas were automatically integrated using Waters® Mass-Lynx software at Target Lynx Program (Waters®). All electrospray ionization and MS parameters were optimised individually for each target compound, and they are listed in Table 2.

2.5. Determination of total phenolics

The total phenolics of samples were determined by the spectrophotometric method (Popova et al., 2007; Ecem-Bayram & Gercek, 2019), with slight modifications. For oral/throat spray analysis, a 25 μL of the defatted phenolic extract, which was previously prepared for the analyses of phenolic profiles, was taken into a clean centrifuge tube and 200 μL Folin-Ciocalteu reagent, 300 μL sodium carbonate solution (20%; w/v) in water, and 1975 μL distilled water were added, respectively. For propolis extract analysis, a 0.5 g of propolis extract (in fine particles) was diluted with 70% ethanol (1:200-fold) and thoroughly shaken. Then, a 25 μL of the diluted propolis was taken into a clean centrifuge tube. Similar procedure was performed as described for defatted phenolic extract.

The same procedure for both samples was performed for calibration solutions at different concentrations [blank, 0.2, 0.4, 0.8, 1.6, and 2.0 mg/mL] prepared using gallic acid stock solution (10 mg/mL). Mixtures were incubated at room temperature for 1 h and the absorbances at 760 nm were measured (Thermo Scientific™ Electron Evolution 300 UV–VIS Spectrophotometer, Waltham, MA, USA). Concentration values obtained from the calibration curve were calculated by multiplying the dilution factor and expressed as gram of gallic acid equivalents (g GAE/L for oral/throat spray and g GAE/kg for propolis extract).

2.6. Determination of cytotoxicity and antiviral activity of oral/throat spray against SARS-CoV-2 infection

Cytotoxicity and antiviral activity against SARS-CoV-2 infection were examined using the xCELLigence real-time cell analyser (RTCA) multiple plates (MP) (Agilent Technologies Inc., Santa Clara, CA, USA) system. The xCELLigence RTCA label-free technology was used to continuous monitoring the cell numbers *via* impedance changes recorded by gold electrodes inserted in patented E-Plates. Once the cells were seeded, their proliferation rate was quantified by tracking the increase in the impedance-related cell index (CI) parameter. The data was collected at 15-minute intervals for a total of 140 h. The cell index time median (CITmed) and cytopathic effect (CPE) delay hours and antiviral activity rate were estimated using the test results (Charretier et al., 2018; Fang, Ye, Wang, Xu, & Reisen, 2011). All samples from the experiment were normalised to the time point when the virus was added. This point was used as normalised cell index (NCI) (Durdagi et al., 2022; Taşlı et al., 2022). In addition, control wells without viruses were used to calculate the antiviral activity rate by subtracting from the CI in the control (virus-only) wells that demonstrated the highest CPE at 40–48 h after adding the virus-containing sample mixture to the cell population. When SARS-CoV-2-induced CPE was entirely or partly suppressed, samples were successively categorised as totally neutralising or partially neutralising. The test sample was considered fully antiviral if it inhibited the SARS-CoV-2-induced CPE at the highest concentration tested. In contrast, it was considered partially antiviral if it was delayed, but did not entirely inhibit CPE at the highest concentration tested (Zost et al., 2020).

2.6.1. Preparation of oral/throat spray

During the preliminary experiments, initial attempts to determine the cytotoxic effect of the oral/throat spray formulation were not successful. Considering this might be due to the higher concentration of black cumin in the composition and its lipophilic character, possibly inducing the decomposition of the cells membrane (data not shown). As black cumin has commonly been recognised as a safe food and natural medicine (Generally Recognized as Safe by the FDA) for centuries (Pelvan et al., 2022; Silva, Haris, Serralheiro, & Pacheco, 2020), the formulation was produced by excluding black cumin oil from the combination before *in vitro* antiviral activity testing. Working samples were

Table 2
MRM transitions and mass spectrometry acquisition parameters of phenolics identified and quantified in propolis extract and oral/throat spray.

Phenolics	Precursor ion (*m/z*)	Product ions (*m/z*) *(Q/q1/q2)	Dwell times (sec.)	Cone voltage (V)	Collision energy (eV) *(Q/q1/q2)	Ionization mode (+/-)	Propolis extract (mg/kg)[a]	Oral/throat spray (mg/L)[b]
Cinnamyl aldehyde	133.2	55.0 / 77.2 / 105.2	0.005	20	15 / 20 / 15	+	–	–
3,4-Dimethoxy benzaldehyde	167.0	124.0 / 139.2	0.005	20	15 / 15	+	–	–
Phloroglucinol	124.9	56.9 / 82.8	0.005	25	15 / 15	–	–	–
4-Hydroxybenzoic acid	137.0	92.9	0.005	20	15	–	–	–
Trans-cinnamic acid	147.0	77.0 / 102.8	0.005	25	20 / 20	–	1,672 ± 150*	45 ± 7*
Protocatechunic acid	152.9	108.8	0.005	25	15	–	–	–
p-Coumaric acid	163.0	93.0 /119.0 / 147.0	0.005	25	20 / 20 / 20	–	1,897 ± 34*	79 ± 2*
2-OH Coumaric acid	163.0	93.0 / 119.0 / 147.0	0.005	25	20 / 20 / 20	–	–	–
m-Coumaric acid	163.0	93.0 /119.0 / 147.0	0.005	25	20/20/20	–	–	–
Phenyllactic acid	165.1	102.8 / 118.9 / 146.9	0.005	25	15 / 15 / 10	–	–	–
Vanilic acid	166.9	90.8 / 108.1 / 123.2 / 152.2	0.005	25	20 / 20 /10 /25	–	–	–
Homogentisic acid	167.0	122.9 / 123.1	0.005	20	20 / 20	–	–	–
Gallic acid	169.0	124.9	0.005	25	20	–	–	–
Shikimic acid	173.0	73.0 / 93.0 / 111.0	0.005	25	20 / 20 / 20	–	–	–
Caffeic acid	179.0	135.0	0.005	25	20	–	1,526 ± 92*	109 ± 7*
Quinic acid	191.1	59.0 / 84.8 / 92.8 / 126.8	0.005	35	20 / 20 / 20/ 20	–	–	–
Ferulic acid	193.0	134.0 / 149.0 / 178.0	0.005	25	20 / 20 / 20	–	1,081 ± 63*	51 ± 0*
Syringic acid	197.0	123.0 / 167.0 / 182.0	0.005	25	20 / 20 / 20	–	–	–
3,4-Dimethoxycinnamic acid	206.7	102.7	0.005	25	20	–	6,073 ± 240*	227 ± 3*
Methylsyringate	211.2	181.0 / 196.0	0.005	25	20 / 20	–	–	–
Resveratrol	227.0	143.0 / 185.0	0.005	30	20 / 20	–	–	–
Chrysin	253.0	151 / 209 / 225	0.005	25	20 / 20 / 20	–	19,024 ± 414*	661 ± 17*
Pinocembrin	255.0	151.0 / 171.0 / 213.0	0.005	25	20 / 20 / 20	–	31,403 ± 1798*	959 ± 64*
Apigenin	269.0	117.3 / 149.0 / 151.0	0.005	40	30 / 25 / 25	–	2,938 ± 203*	101 ± 3*
Genistein	269.0	133.2/ 159.2/ 224.2 /240.0	0.005	40	30 / 20 / 25/ 20	–	466 ± 46*	17 ± 0*
Galangin	269.0	197.0 / 213.0 / 227.0	0.005	25	20 / 20 / 20	–	20,420 ± 722*	648 ± 5*
Naringenin	271.0	145.0 / 151.0	0.005	25	20 / 20	–	2,401 ± 160*	85 ± 4*
Pinobanksin	271.2	153.0 / 225.0 / 253.0	0.005	25	20 / 20 / 20	–	16,248 ± 1116*	544 ± 35*
CAPE	283.0	135.0 / 161.0 / 179.0	0.005	25	20 / 20 / 20	–	13,497 ± 454*	503 ± 0*
Kaempferol	285.0	93.0 / 151.0 / 257.0	0.005	25	20 / 20 / 20	–	3,155 ± 198*	118 ± 8*
Luteolin	285.0	133.0 / 241.0 / 267.0	0.005	25	20 / 20 / 20	–	886 ± 73*	64 ± 29*
Epicatechin	289.1	108.8 / 203.0 / 245.0	0.005	25	20 / 20 / 20	–	–	–
Catechin	289.1	108.8 / 203.0 / 245.0	0.005	25	20 / 20 / 20	–	–	–
Quercetin	301.0	150.8 / 178.9	0.005	35	20 / 20	–	1,145 ± 53*	80 ± 14*
Ellagic acid	301.0	185.2 / 229.0/ 257.0/ 284.2	0.005	30	30 / 25 /25 / 30	–	–	–
Hesperetin	301.3	135.8/ 150.8/ 164.1/ 241.7	0.005	25	20 / 20 / 20/ 20	–	–	–
Taxifolin	303.0	125.0	0.005	25	20	–	–	–
Epigallocatechin	305.2	124.8/ 164.8/ 166.9/ 219.0	0.005	25	20 / 20 / 20/ 20	–	–	–
Isorhamnetin	315.0	300.0	0.005	25	20	–	1,445 ± 108*	58 ± 3*
Myricetin	317.0	137.2 / 151.2 / 179.2	0.005	35	25 / 25 / 20	–	–	–
Chlorgenic acid	353.3	179.0 / 191.0	0.005	25	20 / 20	–	–	–
Rosmarinic acid	359.0	161.0 / 197.0	0.005	25	20 / 20	–	–	–
Rutin	609.1	300.0 / 301.0	0.005	25	20 / 20	–	–	–
Total phenolic content	–	–	–	–	–	–	205 ± 11* (g GAE/kg)	7.91 ± 0.4* (g GAE/L)

Data are expressed as means ± the standard deviation ($n = 3$) on an extract. Statistical significant differences were indicated for each compound ($^*p > 0.05$).
Abbreviations: CAPE, caffeic acid phenethyl ester; GAE, gallic acid equivalents.
[a] Solid.
[b] Liquid.

prepared using 1X medium containing 2% FBS from serial dilutions ranging from 1:160 to 1:10240-fold.

2.6.2. Determination of cytotoxicity of oral/throat spray using RTCA assay

Vero E6 cells were cultured in 1X DMEM supplemented with 10% FBS. The cells were then seeded (25,000 cells/well) in the xCELLigence RTCA MP device (96-well E-plate) to determine the cytotoxicity of oral/throat spray samples. The device was placed at 37 °C with 5% CO_2 humidity incubator throughout the procedure. Seeded cells were maintained in the widget to settle for 24 h. After incubation, diluted samples (from 1:160 to 1:10240-fold) were placed in the xCELLigence RTCA MP device (96-well E-plate). Cells were subsequently incubated with samples at 37 °C for 160 h with 5% CO_2. During this period, the xCELLigence RTCA MP device quantified the electrical impedance into CI with intervals of 15 min. Higher CI values indicated increased cell viability, whereas lower ones suggested poor health and reduced cell viability in the test medium. At the end of the test, obtained data were analysed using a CI value plot of each well against controls *via* RTCA Software Pro 2.6.0 (Basic) (Agilent Technologics, Inc., Santa Clara, CA, USA).

2.6.3. Determination of antiviral activity of oral/throat spray using RTCA assay

High-throughput and quantitative RTCA assay developed with the help of xCELLigence RTCA MP was used to detect the antiviral activity of oral/throat spray samples by measuring the virus's cytopathic effect and the progression of the virus's infection in infected cells. Vero E6 cells were seeded (25,000 cells/well) in sterile, disposable wells of the xCELLigence RTCA MP device (96-well E-plate). Throughout the procedure, the instrument was placed in an incubator and seeded cells were monitored for settling and proliferation for 24 h. The samples were prepared following the protocols described in the "Preparation of oral/throat spray" section. They were incubated at 37 °C for 1 h under 5% CO_2 saturation and with $3.5x10^5$ pfu/mL SARS-CoV-2 virus. After removal of the media from each well, the samples were placed in the wells and incubated at 37 °C for 140 h under 5% CO_2. The cell-only control sample was prepared using untreated wells consisting of only cells and the media containing 2% FBS (state of no infection). While the wells containing only cells and viruses in a medium containing 2% FBS served as a control sample for viruses only (state of infection). Wells holding just cells and sample dilutions were evaluated as a cytotoxicity control for the samples. The apparatus quantified electrical impedance into CI with intervals of 15 min during the whole procedure. Finally, the results were examined using a CI value plot of each well *versus* controls using RTCA Software Pro 2.6.0 (Basic) (Agilent Technologics Inc.). Virus-free control at the same dilution was used in the estimation of antiviral activity percentage of a sample dilution.

2.6.4. Biosafety

All cytotoxicity and antiviral activity experiments were carried out at Biosafety Level 3 (BSL-3) Laboratory of Life Sciences (TÜBİTAK-Marmara Research Center, Gebze-Kocaeli, Turkey), having all necessary national certifications for handling SARS-CoV-2.

2.7. Determination of antimicrobial activity

The antimicrobial assay was performed by the disc diffusion method (Tadtong, Wannakhot, Poolsawat, Athikomkulchai, & Ruangrungsi, 2009). BHIA (Merck KGaA) and 5% SBA (GBL) were used for culturing the *Streptococcus pyogenes*, while TSA (Oxoid) was used for culturing other microbial strains. *S. pyogenes* was cultured in SBA medium under the same condition. The other strains were cultured in TSA at 37 °C for 24 h before being used as inoculum. The grown cultures were washed of the agar using sterile saline solution, and the suspensions were adjusted according to 0.5 McFarland standard turbidity ($1\text{-}5x10^8$ CFU/mL). The antimicrobial screening was performed using BHIA for *S. pyogenes* and TSA for other strains and inoculated with 100 μL of suspension. The inoculum was spread on the agar surface and allowed to dry for 2 h. Each test solution (20 μL) was impregnated on a sterile paper disc of 6 mm diameter and placed on the inoculated agar. The agar plates were then left 30 min at room temperature to allow the diffusion of the test solutions before they were incubated for 24 h at 37 °C. Alcohol, as one of the components in the oral/throat spray, was used as the negative control and a broad-spectrum throat antiseptic chlorhexidine [Chlorhexidine gluconate 0.2% (w/v), Klorhex- DentaSave, Ankara, Turkey] as the positive control. The antimicrobial activity was assessed by measuring the diameter of the inhibition zone (mm) of the test microorganism after incubation. Three independent experiments were performed, and each experiment was run in triplicate. Data were presented as means ± standard deviation.

2.8. Cell culture experiments

2.8.1. Cytotoxicity

Cell viability of each component in oral/throat spray (*e.g.*, each essential oil and propolis extract) and the formulation itself was assessed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Merck KGaA) assay, as described by Erdoğan et al. (2021). RAW264.7 cells at the density of 1×10^5 cells/well were plated in a 48-well plate and treated with different concentrations of essential oils, propolis extract, and oral/throat spray formulation for 24 h. After the incubation period, the cell medium was removed. MTT solution (0.5 mg/mL) was added to all wells and cells were then incubated for an additional 2 h at 37 °C. After incubation, the cell culture medium was discarded and 100 μL of isopropanol was added to wells to dissolve formazan. The absorbance was measured at 570 nm wavelength by an enzyme-linked immunosorbent assay (ELISA) microplate reader (ThermoFisher Scientific, Waltham, MA, USA). Cell viability of cultures treated with samples<70% compared to untreated control cultures (medium group) is considered cytotoxic. The percentage of cell viability was calculated by using the following equation:

$$CellViability(\%) = (Absorbance_{treatmentgroup})/(Absorbance_{control}) \times 100\%.$$

2.8.2. Evaluation of anti-inflammatory activity

Anti-inflammatory activity of each essential oil, propolis extract, and oral/throat spray was evaluated by measuring the nitrite oxide (NO) levels in the cell culture supernatant using Griess reagent (Okur et al., 2020). Briefly, RAW264.7 cells were plated in a 48 well-plate at a density of 1×10^6/mL and incubated for 24 h at 37 °C in 5% CO_2. The cells were pretreated with essential oils, propolis extract, and oral/throat spray at different concentrations and the reference drug indomethacin at 100 μM. Indomethacin was used as a positive control. Two hours later, cells were stimulated with 1 μg/mL of LPS for an additional 22 h. After that, cell culture supernatant was collected. The nitrite concentration in the supernatant was measured using a colorimetric method based on the Griess reaction. The supernatant was mixed with an equal volume of Griess reagent [1% sulfanilamide and 0.1 % N-(1-naphthyl)ethylenediamine dihydrochloride in 5% phosphoric acid] in a 96-well plate for 10 min at room temperature in the dark. The absorbance was read using a microplate reader (ThermoFisher Scientific) at 540 nm. A sodium nitrite standard curve was used to calculate the nitrite

concentration in the samples.

2.8.3. Evaluation of analgesic activity

Prostaglandin E_2 (PGE_2) levels in the collected cell culture supernatants were evaluated using a commercially available quantitative ELISA kit (Abcam PGE2 ELISA Kit, Cambridge, UK), following the manufacturer's instructions. Only doses that showed the highest nitrite inhibition activity for each sample were used.

2.9. Mutagenicity and anti-mutagenicity assays

The standard plate incorporation test was carried out according to the method of Maron and Ames (1983). Mutagenicity assay was performed using *S. typhimurium* TA98 and TA100 strains in the presence and absence of S9 metabolic activation. The sample was diluted with dimethyl sulfoxide (DMSO) and different concentrations (0, 1, 10, 100, 1,000, and 5,000 μg/plate) in 50 μL of DMSO were used for both mutagenicity and anti-mutagenicity assays. The concentration of 5,000 μg/plate was determined as the highest concentration of test samples according to the Organisation for Economic Co-operation and Development (OECD) 471 guidelines (OECD, 1997).

The anti-mutagenicity assay was performed similarly using the same bacterial strains against direct and indirect mutagens. The number of revertant colonies grown on plates containing mutagens without sample was defined as 100%, which means 0% inhibition. The percentage of inhibition was calculated according to a previously described method. The anti-mutagenic effect was considered moderate and strong when the inhibitory effects were 25–40% and over 40%, respectively. The inhibitory effect of<25% was deemed weak and was not recognised as a positive result (Charehsaz, Sipahi, Giri, & Aydin, 2017).

2.10. Statistical analysis

Comparisons among samples were made by using GraphPad Prism 8 (Version 9.4.0, GraphPad Prism, San Diego, CA, USA). Differences among samples were determined by using two-way ANOVA approach. All measurements were carried out in triplicates, unless otherwise stated.

3. Results and discussion

3.1. Development of oral/throat spray formulation

Content and specification of oral/throat spray are given in Table 1. Briefly, an alcoholic mixture of propolis and MCT were mixed with cold-pressed black cumin oil together with α-tocopherol and essential oils from Anatolian sage, oregano, peppermint, lemon peel, and geranium. The ratios of raw materials were selected according to their solubility, taste, antiviral, and anti-microbial effects. Recommended daily amount is 4 to 6 sprays per day for adults and children (11 years of age and older). It is recommended to use 2 or 3 times a day, in the form of 2 sprays.

3.2. Identification and quantification of phenolics in propolis extract and oral/throat spray

Phenolics are valuable secondary metabolites that render various health benefits. Propolis has long been recognised as a rich source of phenolics (Osés et al., 2020). Forty-three natural phenolic compounds were tentatively identified using the upgraded UPLC-MS/MS method; these include 19 phenolic acids, 18 flavonoids and 6 non-flavonoids (aromatic aldehydes (2), ester derivatives (2), stilbenoids (1) and, benzenetriol (1) (Table 2). Out of 43 phenolics, 17 were quantified using authentic standards (Table 2). Among these, pinocembrin (31,403 mg/kg) predominated in propolis extract followed by galangin (20,420 mg/kg), chrysin (19,024 mg/kg), pinobanksin (16,284 mg/kg), CAPE (13,497 mg/kg), and 3,4-dimethoxycinnamic acid (6,073 mg/kg). It was observed that the aforementioned phenolics were also abundant in the final recipe, which especially arose with the addition of propolis extract to oral/throat spray. In this study, black poplar propolis was chosen as the raw propolis type since its CAPE ingredient was relatively high compared to other propolis types. CAPE has been reported to possess various health benefits (Cagli et al., 2005; Kabala-Dzik et al., 2017; Patel, 2016). Propolis offers more than one type of phenolics at the same time.

Total phenolic contents of propolis extract and oral/throat spray were 205 g GAE/kg and 7.91 g GAE/L, respectively (Table 2). The results revealed that the propolis extract used in the oral/throat spray has a sufficient amount of phenolics to fulfil the potential demand for phenolic activity. Remarkable total phenolics measured for spray formulation were able to reflect the possible phenolic-dependent activities of the final product, such as antimicrobial, antiviral, antifungal, anti-inflammatory, and radical scavenging potential (antioxidant).

Due to its richness in phenolics, propolis can suppress the virus replication and modulate the host immune response. It is a common problem that influenza viruses, which cause respiratory tract infections, regularly acquire mutation, making it difficult for successful antiviral therapies (Osés et al., 2020). Recently, the effect of poplar propolis extract (35 μg/mL) on H1N1 (influenza virus) was studied *in vitro,* verifying that it stimulated pro-inflammatory cytokines [interleukins (IL)-6 and IL-1β] secretion by peripheral blood mononuclear cell (PBMC) and reduced neuraminidase enzyme, which is a key protein for virus propagation (Governa et al., 2019). Herpes simplex virus (HSV) infections are also very common health problems worldwide. Huleihel and Isanu (2002) mentioned that propolis hinders the viral absorption and replication cycle of HSV-1 with 0.5% LC_{50} (a lethal concentration that kills half of the cells) in VERO cells, whereas they used a 5% dose for *in vivo* testing in rats and rabbits. Furthermore, Sartori et al. (2012) found that brown propolis (50 mg/kg) reduced the damage caused by HSV-2 infection in BALB/c mice. In the wake of the recent coronavirus outbreak, research on functional products has significantly increased. Since March 2020, in response to the coronavirus pandemic, the South Korean Ministry eased regulations for propolis, which is regarded as a functional food and allowed new oral formulations (Koe, 2020). There is prominent evidence that propolis can diminish and extenuate the symptoms of inflammatory diseases by affecting various metabolic cycles (Hori, Zamboni, Carrão, Goldman, & Berretta, 2013; Machado et al., 2012; Piñeros et al., 2020). Infection by SARS-CoV-2 is characterised by binding between angiotensin-converting enzyme 2 (ACE2) and viral spike protein. Activation of the spike protein is mediated through proteases, such as transmembrane serine protease 2 (TMPRSS2), which play important roles in viral infection (Hoffmann et al., 2020; Wan, Shang, Graham, Baric, & Li, 2021). Two mechanisms can be responsible for the antiviral activity of propolis. Among identified components, CAPE and quercetin are the main ones. CAPE can inactivate serine/threonine-protein kinase (PAK1) directly or upstream, an important enzyme for the entry and replication of several human viruses (Bachevski, Damevska, Simeonovski, & Dimova, 2020; Maruta & He, 2020; Van den Broeke, Radu, Chernoff, & Favoreel, 2010). Quercetin can help against infection by modulating unfolded protein response, preventing the complete viral cycle (Colunga Biancatelli, Berrill, Catravas, & Marik, 2020).

Osés et al. (2020) also reported propolis components (such as catechin, *p*-coumaric acid, and flavonols) displayed an ACE-inhibitory activity. There are numerous evidences for the interference of propolis and/or its components with viral replication and infectivity, potentially reducing lung inflammation owing to anti-inflammatory properties while promoting immune system fortification. These are beneficial properties that could help diminish the symptoms and harmful effects of COVID-19. Propolis has a broad spectrum of effects on bacterial, fungal, or viral infections as an apitherapy product.

3.3. Antiviral activity in oral/throat spray

Before conducting a complete investigation on the antiviral effect of oral/throat spray formulation, the cytotoxicity was evaluated under experimental conditions. Throat spray stock sample and ten different concentrations were prepared as 1/2-fold serial dilutions. Cell index was obtained after monitoring for 140 h *via* the RTCA MP analyser device (Fig. 1A and 1B). The data collected during this period revealed that some concentrations of oral/throat spray samples had a cytotoxic effect on Vero E6 cells. The throat spray dilutions of 1:160 and 1:320 significantly reduced cell viability by over 90% (Fig. 1C) (results were normalised to the time when the virus was added to the experiment). Since both dilutions were considered cytotoxic, samples were deemed non-cytotoxic from 1:640- to 1:10,240-fold dilutions (cell viability percentages were $\geq$ 90%). To properly assess the antiviral efficacy of samples, it was vital to ensure that they were not cytotoxic. Otherwise, it was difficult to determine if a virus or cytotoxicity was responsible for cell death.

Accordingly, the oral/throat spray sample with 1:640-fold dilution provided the highest efficacy (Fig. 2A-2D). The CPE was delayed for 54 h at this dilution and the antiviral activity rate was 85.3%. This result was followed by 1:1280-fold and 1:2,560-fold dilutions which delayed CPE for 24 and 6 h and antiviral activity rates were 22.7 and 1.4%, respectively (Fig. 2C and 2D). In contrast, further reduced dilutions (1:5,120- and 1:10,240-fold) had no antiviral activities on cells (data not shown).

When the antiviral effectiveness data were analysed, it was not possible to identify antiviral efficacy at these dosages since the oral/throat spray was cytotoxic at higher doses. However, after 140 h of incubation with the SARS-CoV-2 virus, the oral/throat spray was not cytotoxic and inactivated 85% of the virus titre when presented at 1:640 dilution.

3.4. Antimicrobial activity in oral/throat spray

The antimicrobial activity of oral/throat spray was evaluated by the disc diffusion method against six common throat pathogen microorganisms (*S. aureus*-MRSA, *S. aureus*-MSSA, *S. pyogenes*, *P. aeruginosa*, *K. pnemoniae*, and *C. albicans*), and the results are summarised in Table 3. Among the tested microorganisms, the oral/throat spray formulation showed strong antimicrobial activity on gram-positive bacteria (*S. aureus*-MRSA, *S. aureus*-MSSA, and *S. pyogenes*) and yeast-like fungus (*C. albicans*), whereas it was inactive against gram-negative bacteria such as *P. aeruginosa* and *K. pnemoniae*. Chlorhexidine, as a positive control, was effective against all tested microorganisms, and the diameter values were within the range of 12–25 mm.

S. aureus, an important pathogen bacteria, causes sore throat and is especially responsible for a wide range of hospital infections worldwide (Kim et al., 2006; Matthews, Adegoke, & Shephard, 2020). As shown in Table 3, oral/throat spray formulation exhibited a larger zone of inhibition against *S. aureus*-MRSA (34 $\pm$ 2.9 mm) and *S. aureus*-MSSA (32 $\pm$ 2.1 mm) when compared to that of chlorhexidine as a positive control (20 $\pm$ 1.2 mm and 19 $\pm$ 1.2 mm, respectively). In addition, the throat spray exhibited the largest inhibitory zone (54 $\pm$ 2.7 mm) against *S. pyogenes*, which is another important respiratory gram-positive pathogen, compared to that of positive control (25 $\pm$ 2.2 mm). When the antifungal effect of the throat spray on *C. albicans* (15 $\pm$ 1.1 mm) was examined, an immediate effect with chlorhexidine (18 $\pm$ 1.2 mm) was observed.

Several studies have been carried out on the antimicrobial effects of black cumin oil, essential oils, and propolis, as the components in the oral/throat spray. In these studies, different magnitudes of effects have been observed depending on the active compounds, extraction methods, concentration, and tested organisms (Ristivojevic et al., 2018; Probst, Sforcin, Rall, Fernandes, & Fernandes Júnior, 2011; Thosar, Basak, Bahadure, & Rajurkar, 2013; Denkova-Kostova et al. 2021; Navit, Margarita, & Liki, 2021; Al-Bakri, Othman, & Afifi, 2010). From the result, it can be concluded that the oral/throat spray formulation exhibited antimicrobial activity against the selected set of microorganisms except for gram-negative bacteria.

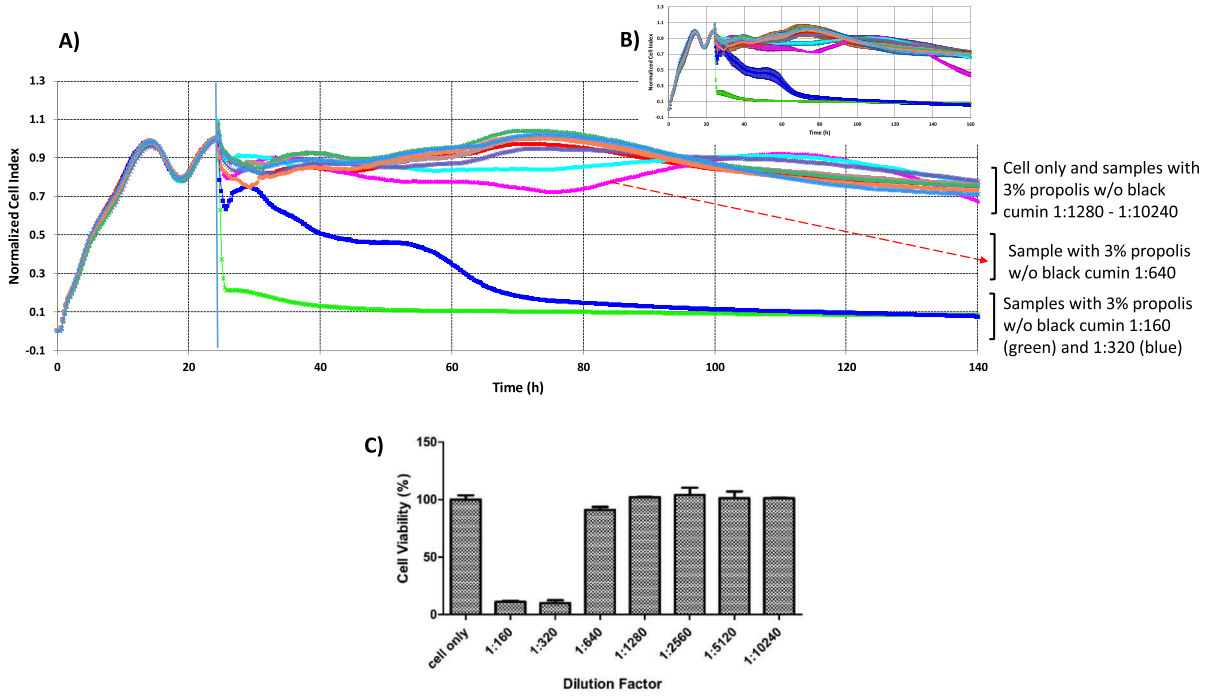

Fig. 1. Cytotoxic effect of oral/throat spray on Vero E6 cell line. **A)** Obtained graph using the xCELLigence RTCA MP real-time cell analysis experiment. Data shows the xCELLigence system's CI. ControlMedia: Vero E6 cells that were not infected with virus (red line) and virus alone; Vero E6 cells that were infected with 3.5x 10^5 PFU/mL of SARS-CoV-2 (B.1.36) (green line). **B)** The second graph depicts the same data but with the standard deviation. **C)** The bar graph shows the cytotoxic effect of samples on the vero E6 cell line. Cell Index data were normalised and renamed NCI based on the time point at when the virus was added to the experiment.

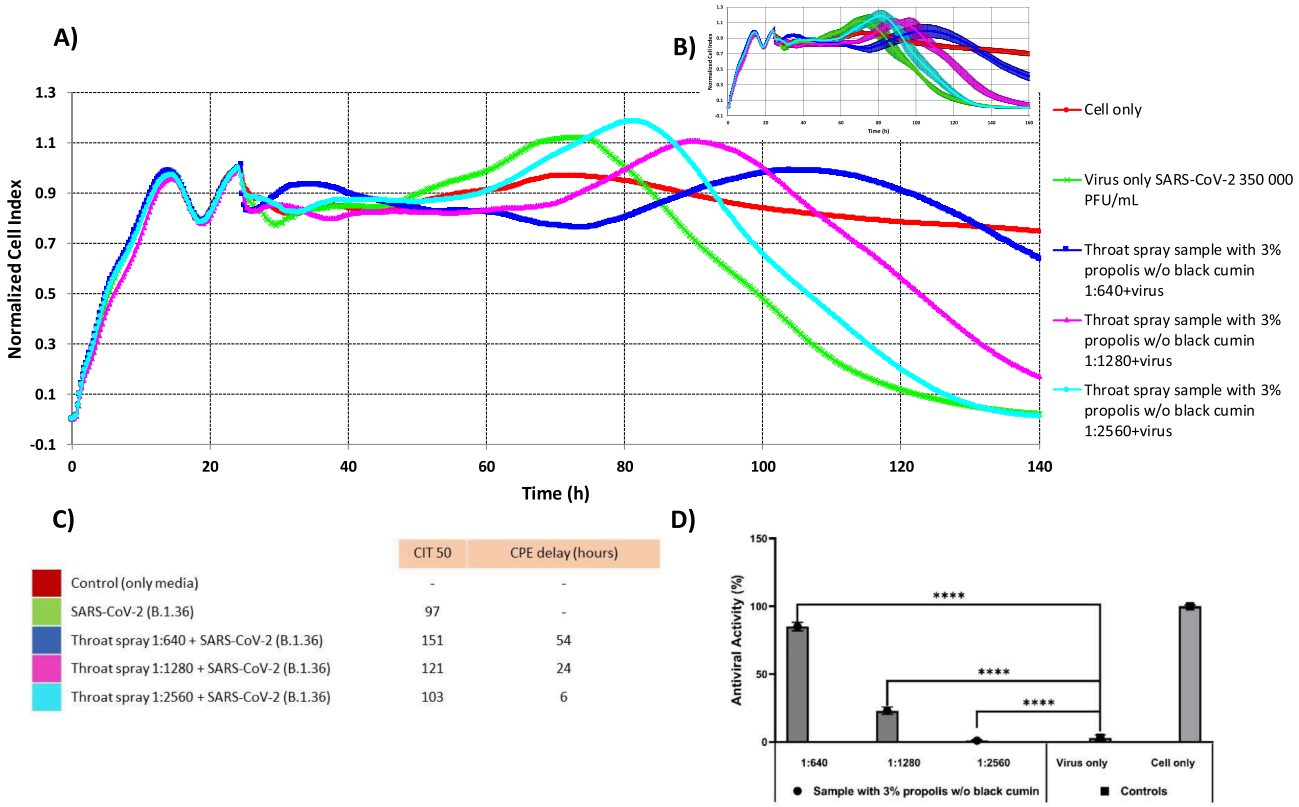

Fig. 2. The graph antiviral activity of the oral/throat spray was evaluated using the xCELLigence RTCA MP real-time cell analysis equipment on the Vero E6 cell line. **A)** The xCELLigence system's CI with the oral/throat spray and control. Control Media: Vero E6 cells that were not infected with virus (red line) and virus alone; Vero E6 cells that were infected with 3.5x 10^5 PFU/mL of SARS-CoV-2 (B.1.36) (green line). The dilution of oral/throat samples. **B)** The second graph depicts the same data but with the standard deviation included. **C)** The table depicted the CITmed and CPE delay hours, respectively. Cell Index data were normalised and renamed NCI based on when the oral/throat spray was added to the experiment. Each curve was obtained from at least four separate duplicates of NCI values. **D)** The bar graph depicted the antiviral activity rate of oral/throat spray. Cell Index data were normalised and renamed NCI based on the time point at when the virus was added to the experiment. After comparing the diluted samples to the control group, the collected data were considered significant, with a p value < 0.0001 (****). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Antimicrobial activity of oral/throat spray and chlorhexidine (positive control).

	Microorganisms	Diameter[a] of inhibition zone (mm)	
		Oral/throat spray	Chlorhexidine (positive control)
G+	*Staphylococcus aureus*-MRSA	34 ± 2.9	20 ± 1.2
	Staphylococcus aureus-MSSA	32 ± 2.1	19 ± 1.2
	Streptococcus pyogenes	54 ± 2.7	25 ± 2.2
G-	*Klebsiella pneumonia*	Ni	12 ± 0.5
	Pseudomonas aeruginosa	Ni	14 ± 1.5
Ylf	*Candida albicans*	15 ± 1.1	18 ± 1.2

Data are expressed as means ± the standard deviation (Three independent experiments were performed and each experiment was run in triplicate).
Abbreviations: G-, Gram negative bacteria; G+, gram positive bacteria; MRSA, Methicillin-resistant *Staphylococcus aureus*; MSSA, Methicillin-susceptible *Staphylococcus aureus*; Ni, No inhibition; Ylf, Yeast-like. fungus.
[a] Includes diameter of disc (6 mm).

3.5. Cell culture studies for activity and safety of oral/throat spray

3.5.1. Cytotoxicity

Cytotoxicity is a measure of an agent's ability to cause cell injury. The ability of all agents to be cytotoxic is dependent on their concentration and exposure time. Events immediately associated with cell death can be measured (e.g., assays of cell viability). Viability assays can be widely applied to determine the cytotoxic potential of an agent (Shaw, 1994).

Prior to the evaluation of anti-inflammatory and analgesic activity, non-toxic concentrations of oral/throat spray and its individual components (such as essential oils, cold-pressed black cumin oil, propolis extract with cell viability of more than 70% were determined. Consequently, cytotoxicity of extracts was carried out on RAW264.7 cells for 24 h by MTT colorimetric assay (Table 4). The selected non-toxic doses for oregano essential oil were 0.0625, 0.125, and 0.25 mg/mL, and the anti-inflammatory and analgesic activity trial continued with these doses.

3.5.2. Anti-inflammatory activity

In vitro anti-inflammatory activities of extracts were assessed by monitoring the decrease in nitrite production levels using the Griess reagent. Essential oils, propolis extract, and oral/throat spray were tested for their inhibitory activities against LPS-induced nitrite production in RAW264.7 cells. In the present study, indomethacin was used as a reference drug (Okur et al., 2021). Upon LPS (1 μg/mL) treatment, nitrite concentration in the cell culture supernatant increased markedly. Various essential oils have been popularly used as anti-inflammatory in aromatherapy (Shen, Jiang, Zhu, & Ou-Yang, 2017). As seen in Table 4, all essential oils showed the capability of reducing LPS-induced nitrite production in a concentration-dependent manner. Anatolian sage and geranium essential oils exhibited the highest anti-inflammatory activity compared to those of others. In particular, the essential oil mixture showed significantly higher anti-inflammatory activity at all doses ($p <$

Table 4
Cell viability (%), nitrite levels, nitrite inhibition capacity, and PGE_2 levels on LPS stimulated RAW 264.7 cell line treated with samples.

Groups	Concentration (mg/mL)	Cell viability (% ± SD)	Nitrite levels (μM ± SD)	Nitrite inhibition capacity (%)	PGE_2 levels (pg/mL)
Control		114.8 ± 2.11	1.87 ± 1.28		43.67 ± 1.78
Control + LPS		100.0 ± 1.26	36.23 ± 1.44		2552 ± 26.62
Indomethacin		96.16 ± 1.87	14.96 ± 1.17*	58.72 ± 2.17	22.55 ± 2.78**
Oregano (*Origanum onites*) essential oil	**0.0625**	84.52 ± 4.19	28.67 ± 1.47*	20.86 ± 4.05	
	0.125	79.11 ± 2.63	27.08 ± 2.28*	25.27 ± 6.30	
	0.25	76.72 ± 2.33	26.13 ± 2.58*	27.87 ± 7.12	16.74 ± 0.52**
	0.5	20.74 ± 2.16			
	1	18.53 ± 1.87			
Anatolian sage (*Salvia triloba*) essential oil	**0.0625**	83.56 ± 2.25	18.38 ± 1.51*	49.27 ± 4.16	
	0.125	75.73 ± 2.76	17.99 ± 2.69*	50.46 ± 5.43	
	0.25	73.74 ± 6.26	12.36 ± 1.15*	65.92 ± 3.17	38.99 ± 8.99**
	0.5	43.04 ± 3.96			
	1	28.83 ± 4.32			
Peppermint (*Mentha piperita*) essential oil	**0.015625**	97.03 ± 2.89	27.98 ± 2.13*	22.77 ± 5.89	
	0.03125	83.98 ± 3.38	25.60 ± 4.76*	29.35 ± 4.13	
	0.0625	79.45 ± 4.64	26.98 ± 1.99*	25.54 ± 5.49	1198 ± 270.2
	0.125	56.61 ± 0.36			
	0.25	52.07 ± 1.31			
	0.5	47.50 ± 1.43			
	1	14.96 ± 2.00			
Geranium (*Pelargonium graveolens*) essential oil	**0.015625**	85.08 ± 3.32	20.29 ± 3.58*	44.01 ± 9.88	
	0.03125	79.97 ± 5.36	21.16 ± 2.64*	55.39 ± 7.28	
	0.0625	74.19 ± 3.74	18.39 ± 1.52*	49.23 ± 4.19	2542 ± 119.2
	0.125	67.39 ± 3.33			
	0.25	42.44 ± 4.59			
	0.5	17.45 ± 3.70			
	1	15.92 ± 3.15			
Lemon (*Citrus limon*) essential oil	**0.125**	83.40 ± 2.92	28.09 ± 1.16*	22.46 ± 3.21	
	0.25	81.29 ± 0.58	27.14 ± 2.57*	25.11 ± 7.08	
	0.5	71.06 ± 2.83	24.25 ± 1.51*	33.06 ± 4.17	212.1 ± 3.32**
	1	15.98 ± 2.43			
Cold-pressed black cumin (*Nigella sativa*) oil	**0.125**	112.4 ± 5.52	23.14 ± 2.56*	36.12 ± 8.62	
	0.25	116.7 ± 1.01	21.14 ± 3.12*	41.66 ± 8.62	
	0.5	110.8 ± 1.90	3.76 ± 1.99*	89.63 ± 5.48	299.8 ± 86.58**
	1	15.44 ± 0.70			
Essential oil mixture	**0.03125**	88.51 ± 3.47	13.63 ± 2.41*	62.38 ± 6.66	
	0.0625	79.98 ± 3.86	7.55 ± 1.13*	79.16 ± 3.13	
	0.125	77.36 ± 4.10	5.85 ± 1.70*	83.85 ± 4.68	874.6 ± 72.81**
	0.25	62.18 ± 2.11			
	0.5	54.02 ± 1.34			
	1	38.76 ± 2.85			
Propolis extract	**0.03125**	102.8 ± 2.14	11.05 ± 7.17*	69.50 ± 4.12	
	0.0625	92.97 ± 3.51	11.94 ± 5.26*	61.04 ± 5.23	
	0.125	91.57 ± 1.96	11.52 ± 3.71*	56.10 ± 1.02	175.0 ± 7.30**
	0.25	67.13 ± 2.15			
	0.5	55.69 ± 3.46			
	1	42.16 ± 2.67			
Oral/throat spray	**0.125**	93.12 ± 1.18	15.90 ± 0.37*	56.10 ± 1.02	
	0.25	84.45 ± 2.10	13.39 ± 1.03*	63.03 ± 2.85	
	0.5	74.13 ± 1.01	10.40 ± 0.60*	71.30 ± 1.66	126.6 ± 1.20**
	1	64.45 ± 2.13			

Values in bold indicate to non-cytotoxic doses of samples. Statistical significant differences were indicated for each compound vs. LPS (*$p < 0.05$,**$P < 0.001$). Abbreviations: L-NAME, Nω-Nitro-L-arginine methylester hydrochloride; LPS, lipopolysaccharides; PGE_2, prostaglandin E_2.

0.05) on LPS stimulated RAW264.7 cells when compared to the control group. Moreover, the percentage inhibition of nitrite at the highest non-toxic dose was relatively high (83.9%) than the reference compound, 100 μM indomethacin (58.7%). The anti-inflammatory activities of these essential oils have been documented in several studies. Previous studies demonstrated that *Thymus vulgaris* (Abdelli et al., 2017), *Salvia officinalis* (Abu-Darwish et al., 2013), *Mentha piperita* (Hejna, Kovanda, Rossi, & Liu, 2021), *Pelargonium graveolens* (Ali, Saleh, & Jalal, 2020), *Citrus lemon* (Shen, Jiang, Zhu, & Ou-Yang, 2017), and *Nigella sativa* (Bordoni et al., 2019) essential oils exhibited anti-inflammatory effects *via* different mechanisms and pathways. Propolis extract exhibited remarkable anti-inflammatory activity compared to the LPS control. Our findings on anti-inflammatory activity of propolis are in agreement with previous studies (Ali, Saleh, & Jalal, 2020). In addition, various concentrations of oral/throat spray (0.125, 0.25, and 0.5 mg/mL) showed significant reductions in LPS-induced NO production (56.10 ± 1.02, 63.03 ± 2.85, and 71.30 ± 1.66%, respectively). Based on these results, the oral/throat spray may be used as an efficient anti-inflammatory agent by inhibiting NO production.

3.5.3. Analgesic activity

PGE_2, a cyclooxygenase (COX) product, is the best-known lipid mediator that contributes to inflammatory pain. Nonsteroidal anti-inflammatory drugs (NSAIDs), primarily PGE_2 inhibitors of COX-1 and/or COX-2, reduce inflammatory pain by reducing prostanoid production (Kawabata, 2011).

PGE_2 levels were determined in LPS (1 μg/mL) stimulated RAW264.7 murine macrophage cells by using an ELISA method. Herein, the

analgesic activity on PGE_2 productions was evaluated for doses of extracts showing the highest anti-inflammatory activity. Indomethacin (100 μM) was used as a positive control in the PGE_2 assay. Table 4 shows that all essential oils, except geranium essential oil, significantly decreased PGE_2 levels more than that of LPS-induced control. Especially, oregano and Anatolian sage essential oils reduced the PGE_2 levels (99.3 and 98.47%, respectively), almost to medium control levels. Previous studies demonstrated that *Thymus vulgaris* (Rašković et al., 2021), *Salvia officinalis* (Qnais, Abu-Dieyeh, Abdulla, & Abdalla, 2010), *Mentha piperita* (Taher, 2012), and *Citrus lemon* (Campêlo et al., 2011) essential oils and *Nigella sativa* (Pop et al., 2020) crude oil showed analgesic activity using different pathways, whereas the analgesic activity of *Pelargonium graveolens* essential oil has not been reported. This is consistent with the present study. In the present study, the propolis extract exhibited similar analgesic activity as seen with the Anatolian sage and lemon essential oils. Moreover, 0.5 mg/mL of the oral/throat spray significantly ($p < 0.05$) suppressed the LPS stimulated PGE_2 production (95%).

3.6. Mutagenicity and anti-mutagenicity assays

The results of the mutagenicity assay showed that the oral/throat spray did not affect bacterial viability, suggesting no cytotoxicity in the tested strains at concentrations up to ≤ 5,000 μg/plate. The results also revealed that the oral/throat spray was not mutagenic at all concentrations tested compared to spontaneous mutation with or without S9 activation (data not shown).

The inhibitory rate percentages of the sample with S9 activation in TA98 and TA100 strains are given in Fig. 3. In the experiment without S9 activation, the oral/throat spray did not show any anti-mutagenic activity against direct mutagens NPD and SA in TA98 and TA100 strains, respectively. The sample showed strong anti-mutagenicity at the dose of 1,000 μg/mL (74% inhibition) and 5,000 μg/mL (99% inhibition) against indirect mutagen 2-aminofluorene (AF) in TA98 strain. In TA100 strain, the inhibition rates of 79 and 103% were observed in 1,000 and 5,000 μg/mL concentrations.

In the anti-mutagenicity assay of this study, the protective effect of the oral/throat spray against the known mutagens in both TA98 and TA100 strains increased after metabolic activation with S9. The effect of metabolic activation on the antimutagenic activity of plant polyphenols was also stated in previous studies (Charehsaz, Sipahi, Giri, & Aydin, 2017). Al-Jenoobi et al. (2010) reported that black cumin seed oil inhibited CYP2D6 and CYP3A4 mediated metabolism of dextromethorphan in human liver microsomes and healthy human volunteers. Such inhibitions may prevent mutagenic/carcinogenic metabolite formation from some pro-carcinogenic chemicals such as B(a)P and aflatoxin B1. Hence, the antimutagenic activity observed in the present study may be attributed to the CYP enzyme inhibition.

4. Conclusions

The oral/throat spray formulation is rich in phenolics (such as pinocembrin, galangin, chrysin, pinobanksin, CAPE, and 3,4 dimethoxy cinnamic acid), essential oils (such as carvacrol, 1,8-cineol, menthol, citral, and geraniol), and cold-pressed black cumin oil (such as nigelline and thymoquinone). Experimental studies have confirmed that the formulation exerted several biological activities i.e. antiviral, antimicrobial, anti-inflammatory, and analgesic. It is noteworthy that after 140 h of incubation with the SARS-CoV-2 virus, the spray was not cytotoxic and inactivated 85% of the virus titre presented at 1:640 dilution. Therefore, this formulation can be used as a dietary supplement not only for the prevention of SARS-CoV-2 infection but also for several other bacterial or viral infections. Further research is required to find out possible benefits of the formulation for individuals infected with SARS-CoV-2 to alleviate the disease complications and effect on the duration of the disease.

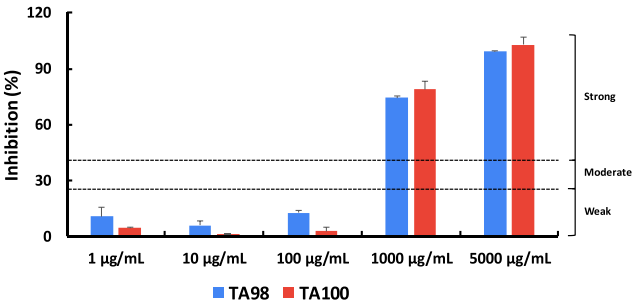

Fig. 3. Anti-mutagenic activity (inhibition %) of the oral/throat against indirect mutagen in TA98 and TA100 strains. The anti-mutagenic activity was considered as follows: **strong**: the inhibitory effect was more than 40%, **moderate**: the inhibitory effect was between 25 and 40%, and **weak**: the inhibitory effect was <25%. The sample showed strong anti-mutagenicity at the dose of 1,000 μg/mL (74% inhibition) and 5,000 μg/mL (99% inhibition) against indirect mutagen 2-aminofluorene (AF) in TA98 strain. In TA100 strain, the inhibition rates of 79 and 103% were observed in 1,000 and 5,000 μg/mL concentrations, respectively. For remaining concentrations, the inhibition rate was below 25%, indicating no antimutagenic activity at these dose levels.

Ethics statement

There are no human clinical trial in this study.

CRediT authorship contribution statement

Ebru Pelvan: Formal analysis, Investigation, Validation, Visualization, Writing – original draft. **Müge Serhatlı:** Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Öznur Karaoğlu:** Formal analysis, Investigation, Validation, Visualization, Writing – original draft. **Bülent Karadeniz:** Formal analysis, Investigation, Validation. **Ceyda Pembeci Kodolbaş:** Investigation, Writing – original draft. **Neşe Aslı Öncü:** Investigation, Writing – original draft. **Gamze Çakırca:** Investigation. **Emel Damarlı:** Funding acquisition, Project administration, Resources, Supervision. **Günay Başdoğan:** Project administration, Resources, Supervision. **Gizem Mergen Duymaz:** Investigation, Resources. **İsmail Emir Akyıldız:** Data curation, Investigation, Methodology, Software, Visualization, Writing – original draft. **Gamze Düz:** Data curation, Formal analysis, Investigation, Software, Validation, Visualization. **Sezer Acar:** Formal analysis, Software, Validation. **Yağmur Özhan:** Investigation, Writing – original draft. **Hande Sipahi:** Investigation, Methodology, Writing – original draft. **Mohammad Charehsaz:** Investigation, Writing – original draft. **Ahmet Aydın:** Writing – original draft. **Erdem Yesilada:** Conceptualization, Supervision, Writing – review & editing. **Cesarettin Alasalvar:** Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Abdelli, W., Bahri, F., Romane, A., Höferl, M., Wanner, J., Schmidt, E., & Jirovetz, L. (2017). Chemical composition and anti-inflammatory activity of Algerian *Thymus vulgaris* essential oil. *Natural Product Communications, 12*, 611–614. https://doi.org/10.1177/1934578X1701200435

Abu-Darwish, M. S., Cabral, C., Ferreira, I. V., Gonçalves, M. J., Cavaleiro, C., Cruz, M. T., Al-bdour, T. H., & Salgueiro, L. (2013). Essential oil of common sage (*Salvia officinalis* L.) from Jordan: Assessment of safety in mammalian cells and its antifungal and anti-inflammatory potential. *BioMed Research International*, *2013*, 538940. https://doi.org/10.1155/2013/538940.

Al-Bakri, A. G., Othman, G., & Afifi, F. U. (2010). Determination of the antibiofilm, antiadhesive, and anti-MRSA activities of seven *Salvia* species. *Pharmacognosy Magazine, 6*, 264–270. https://doi.org/10.4103/0973-1296.71786

Ali, K. M., Saleh, Z., & Jalal, J. (2020). Effect of local propolis irrigation in experimental periodontitis in rats on inflammatory markers (IL-1β and TNF-α) and oxidative stress. *Indian Journal of Dental Research, 31*, 893–898. https://doi.org/10.4103/ijdr.IJDR_909_19

Al-Jenoobi, F. I., Al-Thukair, A. A., Abbas, F. A., Ansari, M. J., Alkharfy, K. M., Al-Mohizea, A. M., … Jamil, S. (2010). Effect of black seed on dextromethorphan *o*- and *n*-demethylation in human liver microsomes and healthy human subjects. *Drug Metabolism Letters, 4*, 51–55. https://doi.org/10.2174/187231210790980435

Bachevski, D., Damevska, K., Simeonovski, V., & Dimova, M. (2020). Back to the basics: Propolis and COVID-19. *Dermatologic Therapy, 33*, Article e13780. https://doi.org/10.1111/dth.13780

Banerjee, A., Kanwar, M., Das Mohapatra, P. K., Saso, L., Nicoletti, M., & Maiti, S. (2021). Nigellidine *(Nigella sativa*, black-cumin seed) docking to SARS CoV-2 nsp3 and host inflammatory proteins may inhibit viral replication/transcription and FAS-TNF death signal *via* TNFR 1/2 blocking. *Natural Product Research, 22*, 1–6. https://doi.org/10.1080/14786419.2021.2018430

Bordoni, L., Fedeli, D., Nasuti, C., Maggi, F., Papa, F., Wabitsch, M., … Gabbianelli, R. (2019). Antioxidant and anti-inflammatory properties of *Nigella sativa* oil in human pre-adipocytes. *Antioxidants, 8*, 51. https://doi.org/10.3390/antiox8020051

Cagli, K., Bagci, C., Gulec, M., Cengiz, B., Akyol, O., Sari, I., … Dinckan, H. (2005). *In vivo* effects of caffeic acid phenethyl ester on myocardial ischemia-reperfusion injury and apoptotic changes in rats. *Annals of Clinical & Laboratory Science, 35*, 440–448. https://doi.org/000232902000011.

Campêlo, L. M., de Almeida, A. A. C., de Freitas, R. L. M., Cerqueira, G. S., de Sousa, G. F., Saldanha, G. B., … de Freitas, R. M. (2011). Antioxidant and antinociceptive effects of *Citrus limon* essential oil in mice. *Journal of Biomedicine & Biotechnology, 678673*. https://doi.org/10.1155/2011/678673

Charehsaz, M., Sipahi, H., Giri, A. K., & Aydin, A. (2017). Antimutagenic and anticlastogenic effects of Turkish black tea on TA98 and TA100 strains of *Salmonella typhimurium (in vitro*) and mice *(in vivo*). *Pharmaceutical Biology, 55*, 1202–1206. https://doi.org/10.1080/13880209.2017.1282969

Charretier, C., Saulnier, A., Benair, L., Armanet, C., Bassard, I., Daulon, S., Bernigaud, B., de Souse, E., R., Gonthier, C., Zorn, E., Vetter, E., Saintpierre, C., Riou, P., & Gaillac, D. (2018). Robust real-time cell analysis method for determining viral infectious titers during development of a viral vaccine production process. *Journal of Virological Methods*, *252*, 57-64. https://doi.org/10.1016/j.jviromet.2017.11.002.

Colunga Biancatelli, R. M. L., Berrill, M., Catravas, J. D., & Marik, P. E. (2020). Quercetin and vitamin C: An experimental, synergistic therapy for the prevention and treatment of SARS-CoV-2 related disease (COVID-19). *Frontiers in Immunology, 11*, 1451. https://doi.org/10.3389/fimmu.2020.01451

Denkova-Kostova, R., Teneva, D., Tomova, T., Goranov, B., Denkova, Z., Shopska, V., … Hristova-Ivanova, Y. (2021). Chemical composition, antioxidant and antimicrobial activity of essential oils from tangerine (*Citrus reticulata* L.), grapefruit (*Citrus paradisi* L.), lemon (*Citrus lemon* L.) and cinnamon (*Cinnamomum zeylanicum Blume*). *Zeitschrift für Naturforschung C, 76*, 175–185. https://doi.org/10.1515/znc-2020-0126

Durdagi, S., Avsar, T., Orhan, M. D., Serhatli, M., Balcioglu, B. K., Ozturk, H. U., … Yumak, Y. (2022). The neutralization effect of montelukast on SARS-CoV-2 is shown by multiscale in silico simulations and combined *in vitro* studies. *Molecular Therapy, 30*, 963–974. https://doi.org/10.1016/j.ymthe.2021.10.014

Ecem-Bayram, N., & Gercek, Y. C. (2019). Appropriate maceration duration for the extraction of propolis. *Fresenius Environmental Bulletin, 28*, 188–192.

Erdoğan, M., Konya, R., Özhan, Y., Sipahi, H., Çinbilgel, İ., Masullo, M., … Kırmızıbekmez, H. (2021). Secondary metabolites from *Scutellaria brevibracteata* subsp. *subvelutina* and their *in vitro* anti-inflammatory activities. *South African Journal of Botany, 139*, 12–18. https://doi.org/10.1016/j.sajb.2021.01.028

Escriche, I., & Juan-Borrás, M. (2018). Standardizing the analysis of phenolic profile in propolis. *Food Research International, 106*, 834–841. https://doi.org/10.1016/j.foodres.2018.01.055

Fang, Y., Ye, P., Wang, X., Xu, X., & Reisen, W. (2011). Real-time monitoring of flavivirus induced cytopathogenesis using cell electric impedance technology. *Journal of Virological Methods, 173*, 251–258. https://doi.org/10.1016/j.jviromet.2011.02.013

Governa, P., Cusi, M. G., Borgonetti, V., Sforcin, J. M., Terrosi, C., Baini, G., … Biagi, M. (2019). Beyond the biological effect of a chemically characterised poplar propolis: Antibacterial and antiviral activity and comparison with flurbiprofen in cytokines release by LPS-stimulated human mononuclear cells. *Biomedicines, 7*, 73. https://doi.org/10.3390/biomedicines7040073

Hejna, M., Kovanda, L., Rossi, L., & Liu, Y. (2021). Mint oils: *In vitro* ability to perform anti-inflammatory, antioxidant, and antimicrobial activities and to enhance intestinal barrier integrity. *Antioxidants, 10*, 1004. https://doi.org/10.3390/antiox10071004

Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., … Pöhlmann, S. (2020). SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell, 181*, 271–280. https://doi.org/10.1016/j.cell.2020.02.052

Hori, J. I., Zamboni, D. S., Carrão, D. B., Goldman, G. H., & Berretta, A. A. (2013). The inhibition of inflammasome by Brazilian propolis (EPP-AF). *Evidence-Based Complementary and Alternative Medicine, 418508*. https://doi.org/10.1155/2013/418508

Huleihel, M., & Isanu, V. (2002). Anti-herpes simplex virus effect of an aqueous extract of propolis. *Israel Medical Association Journal, 4*, 923–927.

Javed, H., Meeran, M. F. N., Jha, N. K., & Ojha, S. (2021). Carvacrol, a plant metabolite targeting viral protease (M^{pro}) and ACE_2 in host cells can be a possible candidate for COVID-19. *Frontiers in Plant Science, 11*, Article 601335. https://doi.org/10.3389/fpls.2020.601335

Kim, J.-S., Song, W., Kim, H.-S., Cho, H. C., Lee, K. M., Choi, M.-S., & Kim, E.-C. (2006). Association between the methicillin resistance of clinical isolates of *Staphylococcus aureus*, their staphylococcal cassette chromosome mec (SCCmec) subtype classification and their toxin gene profiles. *Journal of Microbiology and Infectious Diseases, 56*, 289–295. https://doi.org/10.1016/j.diagmicrobio.2006.05.003

Kabala-Dzik, A., Rzepecka-Stojko, A., Kubina, R., Jastrzebska-Stojko, Z., Stojko, R., Wojtyczka, R. D., & Stojko, J. (2017). Comparison of two components of propolis: Caffeic acid (CA) and caffeic acid phenethyl ester (CAPE) induce apoptosis and cell cycle arrest of breast cancer cells MDA-MB-231. *Molecules, 22*, 1554. https://doi.org/10.3390/molecules22091554

Kawabata, A. (2011). Prostaglandin E2 and pain-an update. *Biological and Pharmaceutical Bulletin, 34*, 1170–1173. https://doi.org/10.1248/bpb.34.1170

Koe, T. (2020). New Rules: South Korea Expands Propolis Oral Formats, Removes Upper Limit For Functional Ingredients. Retrieved from https://www.nutraingredients-asia.com/Article/2020/03/09/. Accessed June 20, 2022.

Machado, J. L., Assunçao, A. K. M., da Silva, M. C. P., dos Reis, A. S. , Costa, G. C., Arruda, D. d. S., Rocha, B. A., Vaz, M. M. d. O. L. L., Paes, A. M. d. A., Guerra, R. N. M., Berretta, A. A., & do Nascimento, F. R. F. (2012). Brazilian green propolis: anti-inflammatory property by an immunomodulatory activity. *Evidence-Based Complementary and Alternative Medicine*, 157652. https://doi.org/10.1155/2012/157652.

Maruta, H., & He, H. (2020). PAK1-blockers: Potential therapeutics against COVID-19. *Medicine in Drug Discovery, 100039*. https://doi.org/10.1016/j.medidd.2020.100039

Matthews, D., Adegoke, O., & Shephard, A. (2020). Bactericidal activity of hexylresorcinol lozenges against oropharyngeal organisms associated with acute sore throat. *BMC Research Notes, 13*, 99. https://doi.org/10.1186/s13104-020-04954-1

Maron, D. M., & Ames, B. N. (1983). Revised methods for the salmonella mutagenicity test. *Mutation Research, 113*, 173–215. https://doi.org/10.1016/0165-1161(83)90010-9

Navit, O.-S., Margarita, Y., & Liki, v. O.-B. (2021). Antimicrobial activity by a unique composition of cold pressed *Nigella sativa* seed (black cumin) oil. *Journal of Food Science and Nutrition Research, 4*, 1–9. https://doi.org/10.33425/2641-4295.1050

Organisation for Economic Co-operation and Development (OECD) (1997). *Test No. 471: Bacterial reverse mutation test*. Paris, France: OECD.

Okur, M. E., Karadağ, A. E., Üstündağ Okur, N., Özhan, Y., Sipahi, H., Ayla, Ş., … Demirci, F. (2020). *In vivo* wound healing and *in vitro* anti-inflammatory activity evaluation of *Phlomis russeliana* extract gel formulations. *Molecules, 25*, 2695. https://doi.org/10.3390/molecules25112695

Okur, M. E., Karadağ, A. E., Özhan, Y., Sipahi, H., Ayla, Ş., Daylan, B., … Demirci, F. (2021). Anti-inflammatory, analgesic and *in vivo-in vitro* wound healing potential of the *Phlomis rigida* Labill. extract. *Journal of Ethnopharmacology, 266*, Article 113408. https://doi.org/10.1016/j.jep.2020.113408

Osés, S. M., Marcos, P., Azofra, P., de Pablo, A., Fernández-Muíño, M.Á., & Sancho, M. T. (2020). Phenolic profile, antioxidant capacities and enzymatic inhibitory activities of propolis from different geographical areas: Needs for analytical harmonisation. *Antioxidants, 9*, 75. https://doi.org/10.3390/antiox9010075

Patel, S. (2016). Emerging adjuvant therapy for cancer: Propolis and its constituents. *Journal of Dietary Supplements, 13*, 245–268. https://doi.org/10.3109/19390211.2015.1008614

Pelvan, E., Karaoğlu, Ö., Fırat, E.Ö., Kalyon, K. B., Eros, E., & Alasalvar, C. (2022). Immunomodulatroy effects of selected medicinal herbs and their essential oils: A comprehensive review. *Journal of Functional Foods, 94*, Article 105108. https://doi.org/10.1016/j.jff.2022.105108

Piñeros, A. R., de Lima, M. H. F., Rodrigues, T., Gembre, A. F., Bertolini, T. B., Fonseca, M. D., … Bonato, V. L. D. (2020). Green propolis increases myeloid suppressor cells and CD4+ Foxp3+ cells and reduces Th2 inflammation in the lungs after allergen exposure. *Journal of Ethnopharmacology, 252*, Article 112496. https://doi.org/10.1016/j.jep.2019.112496

Pop, R. M., Sabin, O., Suciu, Ş., Vesa, S. C., Socaci, S. A., Chedea, V. S., … Buzoianu, A. D. (2020). *Nigella sativa*'s anti-inflammatory and antioxidative effects in experimental inflammation. *Antioxidants, 9*, 921. https://doi.org/10.3390/antiox9100921

Popova, M. P., Bankova, V. S., Bogdanov, S., Tsvetkova, I., Naydenski, C., Marcazzan, G. L., & Sabatini, A.-G. (2007). Chemical characteristics of poplar type propolis of different geographic origin. *Apidologie, 38*, 306–311.

Probst, I. S., Sforcin, J. M., Rall, V. L. M., Fernandes, A. A. H., & Fernandes Júnior, A. (2011). Antimicrobial activity of propolis and essential oils and synergism between these natural products. *Journal of Venomous Animals and Toxins including Tropical Diseases, 17*, 159–167. https://doi.org/10.1590/S1678-91992011000200006

Qnais, E. Y., Abu-Dieyeh, M., Abdulla, F. A., & Abdalla, S. S. (2010). The antinociceptive and anti-inflammatory effects of *Salvia officinalis* leaf aqueous and butanol extracts. *Pharmaceutical Biology, 48*, 1149–1156. https://doi.org/10.3109/13880200903530763

Rašković, A. L., Kvrgić, M. P., Tomas, A. D., Stilinović, N. P., Čabarkapa, V. S., Stojšić-Milosavljević, A.Đ., … Rakić, D. B. (2021). Antinociceptive activity of thyme (*Thymus vulgaris* L.) and interactions with neurotropics and analgesics. *Brazilian Journal of Pharmaceutical Sciences, e18819*. https://doi.org/10.1590/s2175-97902020000318819

Ristivojevic, P., Dimkic, I., Guzelmeric, E., Trifkovic, J., Knezevic, M., Beric, T., … Stankovic, S. (2018). Profiling of Turkish propolis subtypes: Comparative evaluation of their phytochemical compositions, antioxidant and antimicrobial activities. *LWT-Food Science and Technology, 95*, 367–379. https://doi.org/10.1016/j.lwt.2018.04.063

Sartori, G., Pesarico, A. P., Pinton, S., Dobrachinski, F., Roman, S. S., Pauletto, F., … Prigol, M. (2012). Protective effect of brown Brazilian propolis against acute vaginal lesions caused by herpes simplex virus type 2 in mice: Involvement of antioxidant and anti-inflammatory mechanisms. *Cell Biochemistry and Function, 30*, 1–10. https://doi.org/10.1002/cbf.1810

Senthil Kumar, K. J., Gokila Vani, M., Wang, C.- S., Chen, C.- C., Chen, Y.- C., Lu, L.- P., Huang, C.- H., Lai, C.- S., & Wang, S.- Y. (2020). Geranium and lemon essential oils and their active compounds downregulate angiotensin-converting enzyme 2 (ACE2), a SARS-CoV-2 spike receptor-binding domain, in epithelial cells. *Plants (Basel)*, *9*, 770. https://doi: 10.3390/plants9060770.

Shaw, A. J. (1994). Defining cell viability and cytotoxicity. *Alternatives to Laboratory Animals, 22*, 124–126. https://doi.org/10.1177/026119299402200206

Shen, C.-Y., Jiang, J.-G., Zhu, W., & Ou-Yang, Q. (2017). Anti-inflammatory effect of essential oil from *Citrus aurantium* L. var. *amara* Engl. *Journal of Agricultural and Food Chemistry, 65*, 8586–8594. https://doi.org/10.1021/acs.jafc.7b02586

Silva, A. F. C., Haris, P. I., Serralheiro, M. L., & Pacheco, R. (2020). Mechanism of action and the biological activities of Nigella sativa oil components. *Food Bioscience, 38*, Article 100783. https://doi.org/10.1016/j.fbio.2020.100783

Tadtong, S., Wannakhot, P., Poolsawat, W., Athikomkulchai, S., & Ruangrungsi, N. (2009). Antimicrobial activities of essential oil from *Etlingera punicea* rhizome. *Journal of Health Research, 23*, 77–79.

Taher, Y. A. (2012). Antinociceptive activity of *Mentha piperita* leaf aqueous extract in mice. *Libyan Journal of Medicine, 2012*, 7. https://doi.org/10.3402/ljm.v7i0.16205

Taşlı, N. P., Gönen, Z. B., Kırbaş, O. K., Gökdemir, N. S., Bozkurt, B. T., Bayrakcı, B., … Cetin, M. (2022). Preclinical studies on convalescent human immune plasma-derived exosome: Omics and antiviral properties to SARS-CoV-2. *Frontiers in Immunology, in press,*. https://doi.org/10.3389/fimmu.2022.824378

Thosar, N., Basak, S., Bahadure, R. N., & Rajurkar, M. (2013). Antimicrobial efficacy of five essential oils against oral pathogens: An *in vitro* study. *European Journal of Dentistry, 7*, 71–77. https://doi.org/10.4103/1305-7456.119078

Valussi, M., Antonelli, M., Donelli, D., & Firenzuoli, F. (2021). Appropriate use of essential oils and their components in the management of upper respiratory tract symptoms in patients with COVID-19. *Journal of Herbal Medicine, 100451*. https://doi.org/10.1016/j.hermed.2021.100451

Van den Broeke, C., Radu, M., Chernoff, J., & Favoreel, H. W. (2010). An emerging role for p21-activated kinases (Paks) in viral infections. *Trends in Cell Biology, 20*, 160–169. https://doi.org/10.1016/j.tcb.2009.12.005

Wan, Y., Shang, J., Graham, R., Baric, R. S., & Li, F. (2020). Receptor recognition by the novel coronavirus from Wuhan: An analysis based on decade-long structural studies of SARS coronavirus. *Journal of Virology*, e00127–20. https://doi.org/10.1128/JVI.00127-20

Wani, A. R., Yadav, K., Khursheed, A., & Rather, M. A. (2021). An updated and comprehensive review of the antiviral potential of essential oils and their chemical constituents with special focus on their mechanism of action against various influenza and coronaviruses. *Microbial Pathogenesis, 152*, Article 104620. https://doi.org/10.1016/j.micpath.2020.104620

Yosri, N., Abd El-Wahed, A. A., Ghonaim, R., Khattab, O. M., Sabry, A., Ibrahim, M. A. A., … El-Seedi, H. R. (2021). Antiviral and immunomodulatory properties of propolis: Chemical diversity, pharmacological properties, preclinical and clinical applications, and in silico potential against SARS-CoV-2. *Foods, 10*, 1776. https://doi.org/10.3390/foods10081776

Zost, S. J., Gilchuk, P., Chen, R. E., Case, J. B., Reidy, J. X., Trivette, A., … Crowe, J. E., Jr (2020). Rapid isolation and profiling of a diverse panel of human monoclonal antibodies targeting the SARS-CoV-2 spike protein. *Nature Medicine, 26*, 1422–1427. https://doi.org/10.1038/s41591-020-0998-x