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Original Article

SARS-CoV-2 neutralizing activity of polyphenols in a special green tea extract preparation

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A R T I C L E  I N F O

Keywords:
SARS-CoV-2 Virus neutralization assay Green tea Epigallocatechin-3-O-gallate Throat spray Mucosa

A B S T R A C T

Background: The COVID-19 pandemic will continue to threaten our health care systems in the next years. In addition to vaccination there is a need for effective tools for prevention and treatment. Products from natural sources, like standardized plant extracts offer a wide range of antiviral effects and possible applications. Purpose: The aim of this study was to investigate, whether a sorbitol/lecithin-based throat spray containing concentrated green tea extract (sGTE) interacts with SARS-CoV-2 viral particles and additionally is capable to block the virus replication.

Study design and methods: The antiviral effect was studied in a VeroE6 cell culture model, including concentration/effect correlations and the biological mechanism of virus blockade, using the Wuhan type of SARS CoV-2 as well as its beta- and delta-mutations. In addition, the qualitative and quantitative tannin profile present on the oral mucosa after spray application has been investigated by LC-MS/MS and HPLC-DAD analyses of (-)-epigallocatechin-3-O-gallate (EGCG) and related catechin derivatives.

Results: The findings of this study demonstrate, that sGTE has strong neutralizing activity on SARS-CoV-2, resulting in an up to 6,3E+04-fold reduction of infectivity independent from the strain. The type of interaction of sGTE with surface proteins seems to be direct and non-specific concerning the viral surface protein structures and resembles the general non-specific activity of polyphenols. By HPLC-DAD analysis, eight catechins were identified in sGTE, with EGCG and (-)-epicatechin-3-O-gallate as the most abundant ones. The total content of catechin derivatives, calculated as catechin, was 76 g/100 g. LC-MS/MS and HPLC-DAD analyses of throat swabs after application of a sGTE spray have shown that the concentrations of green tea tannins in the pharyngeal mucosa are higher than the effective dose found in the in vitro studies with SARS-CoV-2, even 1 h after the last application.

Conclusion: The findings of this study suggest that sGTE has strong neutralizing activity on SARS-CoV-2 independent from the strain (Wuhan strain, beta- or delta-variants). sGTE might be relevant for reduction of cor

Abbreviations: BSL-3, Bio-Safety Level III; CRS, Chemical Reference Substance; DAD, Diode Array Detector; EGCG(-), -epigallocatechin-3-O-gallate; FSC, fetal calf serum; GTE, special green tea extract; HPLC, High Performance Liquid Chromatography; HESI, heated electrospray ionization; HPTLC, High Performance Thin Layer Chromatography; HRS, Herbal Reference Substance; IHC, Immunohistochemical staining; ISTD, internal standard; LC-MS/MS, liquid chromatography – mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MA, metabolic activity; MEM, Minimum essential medium; MOI, Multiplicity of infection; PBS, Phosphate buffered saline; PenStep, Penicillin- Streptomycin; PFU, Plaque forming units; qRT-PCR, Quantitative real-time polymerase chain reaction; R, reagent grade according to European Pharmacopoeia; RC, regenerated cellulose; RFU, relative fluorescent unit; rMA, relative metabolic activity; RNA, Ribonucleic acid; RSD, relative standard deviation; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus-2; sGTE, special green tea extract preparation (spray); TCID50, Tissue Culture Infection Dose 50; UHPLC-HRMS, Ultra high performance liquid chromatography – high resolution mass spectrometry; w/o, without.

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https://doi.org/10.1016/j.phymed.2022.153970
Received 31 October 2021; Received in revised form 20 January 2022; Accepted 27 January 2022
Available online 30 January 2022
Introduction

The current COVID-19 pandemic caused more than 240 million infections worldwide during the past two years (https://covid19.who.int/) and will continue to threaten our health care systems in the next years. The disease is caused by SARS-CoV-2, a single stranded RNA β-coronavirus with a most likely zoonotic origin that is transmitted from human to human via aerosols and respiratory droplets (Hu et al., 2021). The virus enters human cells mainly by binding to a membrane protein that regulates the renin-angiotensin system (angiotensin converting enzyme 2 - ACE2) and uses the serine protease TMPRSS2 for spike (S) protein priming (Hoffmann et al., 2020). Immunohistochemical studies revealed that ACE2 is abundantly expressed in the ciliated epithelial cells (especially within the motile cilia), as well as in alveolar type II (AT2) cells in the lung alveoli. Since TMPRSS2 protein is also expressed in those cell types, it is likely that they represent the early entry site for coronaviruses (Lee et al., 2020).

Current approaches in the search for possible treatments for COVID-19 include antivirals, immunomodulatory agents, and immunoglobulins (Kyiakidis et al., 2021; Prijsters et al., 2020). Substances that occupy the host ACE2 receptor or TMPRSS2 may be capable of preventing virus entry into the cell (Sanders et al., 2020). The development and authorization of vaccines against the SARS-CoV-2 virus certainly depict a milestone in the fight against COVID-19. Recently, it has been argued that vaccination alone will be insufficient to contain the outbreak (Storozhuk, 2021). Developing a tannin-based throat spray, which according to European Pharmacopeia, dried, non-fermented leaves of *Hamamelis virginiana* were described as having antiviral activities against SARS-CoV-2 and influenza viruses has been demonstrated from natural sources, like plant extracts and their constituents offer a wide range of antiviral effects and possible applications (Hensel et al., 2020).

Antiviral activities of tannins against various viruses like enterovirus, caliciviruses, rotavirus, HIV, herpes simplex virus, and coronaviruses, have intensively been investigated. Already Erdelmeier et al., (1996) reported the antiviral activities of tannin-containing extracts from *Hamamelis virginiana* bark agents against Herpes simplex virus and HIV. Recently the antiviral activity of tannin-containing plant juices against SARS-CoV-2 and influenza viruses has been demonstrated in vitro (Frank et al., 2020).

A recent study showed that corilagin, a galloylated ellagitannin, which can be found in many plants such as *Dimocarpus longan*, *Phyllanthus urinaria*, and *P. emblica*, prevents SARS-CoV-2 infection by targeting RBD-ACE2 binding (Yang et al., 2021).

Leaves of *Camellia sinensis* (L.) Kuntze (Theaceae) contain catechin and epicatechin derivatives including epigallocatechin-3-O-gallate (EGCG) as tannins. Xin et al., (2018) reported at least 75 chemical substances including catechins, flavonoids etc. in green tea leaves. According to European Pharmacopoeia, dried, non-fermented leaves of *C. sinensis* should contain at least 8.0% of total catechins, expressed as EGCG. Specific extracts from these leaves usually have a catechin content of 60 - 80%.

Recently, it has been shown that extracts from black and green tea rapidly inactivate SARS-CoV-2 in saliva in vitro (Ohgitani et al., 2021), and that EGCG from green tea effectively blocks infection of SARS-CoV-2 and new variants by inhibiting spike protein binding to ACE2 receptor (Liu et al., 2021a). It has also been discussed whether green tea consumption is correlated with lower COVID-19 morbidity and mortality in certain countries (Storozhuk, 2021).

Since SARS-CoV-2 viruses accumulate in epithelial cells of the saliva glands and oral mucosa (Huang et al., 2021), it has been suggested to use mouth-rinses for reducing the viral load (Lloyd-Jones et al., 2021; Sen-eviratne et al., 2021). Developing a tannin-based throat spray, which prevents the entry and builds a mechanical thin layer against viral attachment, would be an elegant and non-invasive possibility to prevent infections.

Therefore, the aim of this study was to investigate, whether a sorbitol/lecithin-based throat spray, containing concentrated green tea extract, interacts with SARS-CoV-2 viral particles and, additionally whether it is capable to block the virus replication. The antiviral effect was studied in a VeroE6 cell culture model, including concentration/ effect correlations and the biological mechanism of virus blockade, using the Wuhan type of SARS-CoV-2, beta- and delta-mutations. In addition, the qualitative and quantitative tannin profile present on the oral mucosa after spray application has been investigated by LC-MS/MS and HPLC-DAD analyses of EGCG and related catechins.

Material and methods

Chemicals

The chemicals used were reagent grade according to the European Pharmacopoeia (indicated as “F”) unless otherwise mentioned. Acetonitrile for chromatography R, methanol R and water for chromatography R were from Promochem (Wesel, Germany), anhydrous formic acid R, ascorbic acid R, caffeine, (ethylenedinitrilo)tetraacetic acid R and methanol R were from Merck (Darmstadt, Germany). (+)-Epigallocatechin-3-O-gallate CRS (Chemical Reference Substance; code Y001936, purity: 93%), benzanilide (code 32,434, supplier batch S29552-386) and green tea dry extract HRS (Herbal Reference Substance) were from EDQM (Strasbourg, France). Water R was produced in an in-house water purification system. Special green tea leaf dry extract (GTE) and a mouth and throat spray containing 8% GTE (SGTE) were provided by Phytovisions (Garmisch-Partenkirchen, Germany).

High performance liquid chromatography diode array detection (HPLC-DAD) analysis of GTE

The HPLC method was based on Monograph 10.0/2668 for green tea of European Pharmacopoeia. For peak identification, the DAD-spectra and the chromatogram of a solution of green tea dry extract HRS were compared with the corresponding exemplary chromatogram (https://extranet.edqm.eu/4DLink1/pdfs/chromatos/2668.pdf). Furthermore, the relative retention times of the Monograph of United States Pharmacopeia of powdered decaffeinated green tea extract were used. The preparation of the solutions and the methods are described below.

Sample solution: Approximately 200 mg GTE, accurately weighed, were dissolved in 20 ml methanol 70% (v/v) and sonicated for 10 min. It was diluted with stabilizing solution to a final concentration of 0.5 mg/ml and filtrated through a membrane (RC, 0.45 µm).

Reference solution: A stock solution of EGCG CRS and caffeine in water R (0.8 mg/ml each) was diluted with stabilizing solution to a final concentration of 32 µg/ml each. The solution was filtrated through a membrane (RC, 0.45 µm) prior to use.

Stabilizing solution: Approximately 250 mg (ethylenedinitrilo)tetraacetic acid R and 250 mg ascorbic acid R, accurately weighed, were solved in water R to a final volume of 500 ml. After adding 100 ml acetonitrile for chromatography R it was filled up with water R to a final volume of 1000 ml.

HPLC analyses were performed using an Agilent 1260 liquid chromatograph (Agilent technologies, Waldbronn, Germany). Samples were separated on a YMC-Pack ODS-A (150 × 4.6 mm, 3 µm, 12 nm, YMC Co., LTD., Kyoto, Japan). The injection volume was 10 µl. The mobile phase consisted of water for chromatography R containing 0.05% (v/v) anhydrous formic acid R (A), methanol R containing 0.05% (v/v) anhydrous formic acid R (B) and acetonitrile for chromatography R (C). A gradient program was used as follows: 0–5 min., 97% A/0% B/3% C; 5–23 min., 97–67% A/0–30% B/3% C; 23–29 min., 67% A/30% B/3% C; 29–30 min, 67–30% A/30–67% B/3% C; 30–36 min., 30% A/67% B/3% C; 36–37 min., 30–97% A/67–0% B/3% C; 37–42 min., 97% A/0%
B/3% C. The flow rate was 0.8 ml/min. The column temperature was 25 °C. The DAD detector scanned from 190 to 450 nm, and the samples were detected at 210 nm (for qualitative experiments) and 278 nm (for quantitation).

**Size exclusion chromatography (SEC) analysis of GTE**

Approximately 1 g GTE, accurately weighed, was dissolved in water to a final volume of 50 ml. Every 10 min. the solution was gently mixed during 60 min. Prior to use, it was filtrated through a membrane (RC, 0.45 μm).

SEC analysis was performed using an Agilent 1260 liquid chromatograph. Samples were separated on two inline PL aquagel-OH MIXED-H (300 × 7.5 mm, 8 μm, Agilent technologies). The injection volume was 100 μl. The isotropic separation for 30 min. at a flow rate of 1.0 ml/ml was done with water R as mobile phase. The column and detector temperature were 40 °C.

Molecular weight calibration was done with aqueous solutions (0.5 mg/ml) of 10 different pullulans (Pullulan polysaccharide calibration kit, Agilent technologies).

**Analysis of catechins in throat swabs after pharyngeal application of sGTE mouth and throat spray**

**Study protocol**

The study protocol was approved by the Ethics Commission at University of Graz (reference number 39/137/63 ex 2020/21). The written declaration of consent was in accordance with the Helsinki Declaration of 1975.

Six healthy adults (4 females, 2 males, aged 28 – 65 years) were enrolled. 15 min. prior to the first application of sGTE, the volunteers were asked to drink a glass of water. 5 min. prior to spray application, a control throat swab was taken from the pharyngeal region of each participant. At 0, 30, 60, 90 and 120 min., two puffs of the preparation were applied to the pharynx. Immediately (1 min.) after applying the first sGTE dose, a pharyngeal swab was taken; subsequently, swabs were taken right before the application of the next sGTE dose (at approx. 27, 57, 87 and 117 min.); two further swabs were taken 30 and 60 min. after the last sGTE dose (i.e. 147 and 177 min. after the first dose).

The throat swabs were immediately transferred to polypropylene tubes containing 1 ml solution of benzamidine in 80% methanol (14 μg/ml) that was used as internal standard (ISTD) and extracted in an ultrasonic bath for 15 min. Thereafter, the samples were centrifuged (4 °C, 16,000 x g, 10 min.), and the supernatants were analysed by LC-MS for the catechin fingerprint and by HPLC-UV for determination of the EGCG content. For determination of the salvia amounts on the throat swabs, participants were asked to take three swabs without sGTE spray application and to weigh the swabs before and after throat swab.

**Liquid chromatography-mass spectrometry (LC-MS/MS) analysis of catechin fingerprint**

Analyses were performed on an Ultimate 3000 HPLC system hyphenated to a LTQ-XL mass spectrometer (Thermo Scientific, Waltham, MA, USA). As a stationary phase, an Acquity BEH C18 column (1.7 μm, 2.1 × 150 mm, Waters Corp, Milford, MA, USA), equipped with a VanGuard™ precolumn (BEH C18, 1.7 μm, 2.1 mm x 5 mm, Waters Corp.) was used. Chromatographic conditions were as described by Xin et al., (2018). The mobile phase consisted of H₂O+0.1% formic acid (A) and acetonitrile +0.1% formic acid (B), and the following gradient was used: 0–1 min., 5–7% B in A; 1–4 min., 7–13% B in A; 4–20 min., 13–28% B in A; 20–25.5 min., 28–100% B in A; 20.5–26 min., 100% B; 26–26.5 min., 100–5% B in A; 26.5–32 min., 5% B in A; column temperature was 35 °C and flow rate was 0.25 ml/min. The MS was run in the heated electrospray ionization (HESI) negative mode, and the sensitivity of the ion optics was optimized to EGCG (m/z 457). Heater temperature was 330 °C, capillary temperature was 250 °C, sheath gas flow was 27 arbitrary units, source voltage was -3 kV, capillary voltage was -31 V, and tube lens was -98 V.

In addition to the throat swab samples, a 1:1800 dilution of the sGTE preparation with 80% methanol was prepared for analysing the catechin fingerprint in the preparation itself.

**EGCG quantification by HPLC-DAD**

Measurements were performed on a Vanquish Core™ quaternary HPLC system equipped with a DAD (Thermo Scientific) using the same stationary phase, mobile phase, flow rate and column temperature as for the LC-MS/MS analyses. The gradient was adjusted to allow baseline separation of EGCG: 0–12 min, 8% B in A; 12–12.5 min, 8–60% B in A; 12.5–20.5 min, 60–100% B in A; 20.5–22 min, 100% B; 22–22.5 min, 100–8% B in A; 22.5–28 min, 8% B in A). The UV detection wavelength was 274 nm. To compensate potential dilution effects (e.g by saliva from the throat swabs), the peak area of EGCG was normalized by dividing by the peak area of the ISTD contained in the extraction solvent.

For determination of linearity, a dilution series of EGCG (9 concentrations, 0.09–186 μg/ml) in 80% CH₃OH containing 14 μg/ml ISTD was analysed, and the calibration curve was established by linear regression analysis. Limits of quantitative and detection (LOQ and LOD) were determined based on the signal-to-noise ratio detected in EGCG dilutions. Accuracy was determined by analysing EGCG test solutions containing 27.2, 54.4 and 81.6 μg/ml EGCG. Precision was determined by assessing intraday and inter-day variation. Intraday variation was determined by analysing one sample containing 69.7 μg/ml EGCG nine times within one day. Inter-day variation was determined by analysing the same sample for three times on three different days. Extraction recovery of EGCG from the throat swabs was determined by taking throat swabs without application of sGTE and spiking these control swabs with defined amounts of EGCG (27.2, 54.4 and 81.6 μg). The spiked swabs were extracted in the same way as described in the study protocol, and the recovered EGCG concentrations were compared with that of the directly prepared respective EGCG dilutions that were also used for accuracy determination. Results are expressed as mean ± SD.

**Cell culture and virus stock preparation**

African green monkey kidney epithelial cells VeroE6 from Biomedica (Vienna, Austria; VC-FTV6) were maintained in Gibco’s Minimum Essential Medium supplemented with Earle’s Salts and L-Glutamine (Gibco, Waltham, MA, USA; 11,095–080, 500 ml) with 5% fetal calf serum (FCS) from Gibco and 1% PenStrep, in the following referred to as MEM 5%. Incubation at 37 °C, 5% CO₂ if not stated otherwise. In the following incubation at 37 °C, 5% CO₂ is abbreviated as incubation.

The human 2019-nCoV isolates (Ref-SKU: 026V-03883 (Wuhan Strain) and Ref-SKU: 014V-04058 (B.1.351) from EVAg) were proliferated in VeroE6 cells, TCID50 titres were determined according to the Reed Munch method (Ramakrishnan, 2016). PFU was calculated with the conversion factor 0.7, based on the ATCC-LGC Standards (www.atcc.org/support/technical-support/faq/convertong-tcld-50-to-plaque-for-ming-units-pfu). The delta variant derived from a patient was sequenced, characterized, and propagated using the same procedure as the viruses from EVAg (sequence information: www.gisaid.org Accession ID: EPI_ISL_4,847,176). For all infection experiments, the working stocks were diluted to a calculated multiplicity of infection (MOI) 0.002 in MEM 2%. All experimental steps with active SARS-CoV-2 virus isolates were performed under BSL-3 conditions.

**Substance preparation: sGTE**

The formulated sGTE was diluted in MEM 2% to substance concentrations of 1:450, 1:600, 1:900, 1:1800 and 1:4500, respectively. Substance dilutions were prepared sterile and freshly prior to every assay. All following descriptions of “certain concentrations / dilutions of sGTE”
refer to those dilutions.

Metabolic activity assay

For metabolic activity (MA) assays VeroE6 cells were seeded in 48-well cell culture plates with a density of 3.0E+04 cells per well. Cells were seeded in MEM supplemented with 2% FCS, 24 h before substance treatment. Incubation at 37 °C, 5% CO₂.

After incubation, the cells were exposed to certain dilutions of sGTE in MEM + 2% FCS (as described above). Triplicates per substance and concentration were tested. Two protocols were applied: 1) After 24 h incubation, cells were washed twice with MEM without (w/o) supplements, the metabolic activity was measured using a resazurin-based assay. 2) Cells were incubated for 1 h. After a washing step, cells were treated with MEM 2% and incubated for further 24 h before viability measurement. After the addition of Resazurin (10 µM concentration), the increase of relative fluorescent units (RFU) was measured for 2 h and subsequently a linear regression analysis of gained data was performed. Slopes from substance treated cells were compared to the untreated controls. In addition, slopes were normalized to that of untreated controls (untreated cells = cells + respective amount of medium) to calculate the relative metabolic activity in percent.

Virus neutralization assay

VeroE6 cells were seeded with a density of 3.0E+04 cells per well in a 48-well plate (Corning Costar, Corning, NY, USA), cell culture treated in 300 µL MEM + 2% FCS, 24 h prior to the virus neutralization assay.

At the infection day, the seeding medium was removed and the cells were either pre-treated 2 h with medium (MEM 2% FCS) containing sGTE in certain concentrations or medium change with or w/o sGTE was made directly before viral infection, depending on the treatment protocol (Table 2). Based on this protocol, either cells, virus (1 h at 37 °C with continuous slow shaking) or both were treated with sGTE in certain concentrations. During 1 h infection with virus diluted to a calculated MOI 0.002, cells were kept at 37 °C and 5% CO₂. After that, the infection medium was removed, and the cells were washed twice with MEM w/o supplements to remove unbound virus. Subsequently, 300 µL fresh MEM + 2% FCS, either with or w/o sGTE (as given in Table 2) were given on the cells. The cells were incubated for additional 24 h at 5% CO₂ and 37 °C until 140 µL supernatant was harvested and inactivated to extract RNA and quantify the viral copies numbers via qRT-PCR. Untreated infected cells serve as positive controls in the assay, no substances to lower or inhibit the viral replication were used in these controls. Non-infected cells serve as the background of the assay, no substance and no virus were used in the negative controls. Serum from patients infected cells serve as the background of the assay, no substance and lower or inhibit the viral replication were used in these controls. Non-infected cells served as the background of the assay, no substance and no virus were used in the negative controls. Serum from patients recovered from COVID-19 (infected in 2020 and 2021) were used under application of the same protocol procedures as neutralizing efficiency controls (in vitro use of serum was approved by the Ethics Commission at Medical University Graz (reference number 33–195 ex 20/21; including sub-study: COV-BB-001)). In addition, the 48-well plate was fixed in 4% formalin for SARS-specific immunohistochemical staining (IHC).

RNA isolation, qRT-PCR and determination of viral copy numbers

After inactivation of the supernatant samples containing virus with AVL buffer (Qiagen, Hilden, Germany), the viral RNA was isolated using the Qlamp viral RNA mini Kit following the manufacturer’s protocol. The RNA was eluted in 40 µL ultra-pure H₂O and stored at -80 °C.

The viral RNA was amplified with the CDC recommended primers and probe set of N1 and N2 from 2019-Novel Coronavirus (2019-nCoV) Real-time RT-PCR Panel (www.cdc.gov/coronavirus/2019-ncov/lab/rt-pcr-panel-primer-probes.html) using a QuantiTect Multiplex RT-PCR Kit (Qiagen) with a Rotor Gene Q cycler. The reaction volume was reduced to 25 µL, with amplification for 30 min. at 50 °C and 15 min. at 95 °C, the steps was followed by 45 cycles (95 °C for 3 min. and 55 °C for 30 s). The sequence of the used primers and probes can be found in Table 1.

For the calculation of viral copy numbers, a commercially available copy number standard (ATCC VR-1986D genomic RNA from 2019 Novel Coronavirus, Lot: 70,035,624) was serially diluted and analysed via qRT-PCR. The resulting cq-values were plotted against ln(copy numbers) and the equation received from linear regression analysis was used to calculate the viral copy numbers from the cq-values of the samples for Primer and Probe N1 and N2, respectively. Regression curves for N1: y = -1.442 x + 35.079 and N2: y = -1.5 x + 38.357. These calculated viral copy numbers refer to a volume of 5 µL RNA eluate, used as template in qRT-PCR.

Immunohistochemistry (IHC)

After fixation of the cells with 4% formalin in the 48-well cell culture plate and washing with PBS, the cells were permeabilized using 0,1% Triton X 100 in PBS for 10 min (200 µL per well), followed by 3 washing steps with PBS. The endogenous peroxidases were blocked with 3% H₂O₂ in methanol for 30 min. After this, 3 washing steps with 200 µL PBS followed and the cells were incubated for 1 h with 100 µL of a 1:1000 dilution of primary antibody (SARS-CoV-2 (2019-nCoV) Nucleocapsid Antibody, Rabbit, Mbio, Sinobiological Cat: 40,143-R019) in antibody diluent (REAL Antibody diluent, Agilent Technologies, Dako Cat: S202230-2) per well. Three washing steps with PBS followed and cells were treated with the secondary antibody (EnVision™ + Dual Link System HRP, Agilent Technologies, Dako Cat: K5007) for at least 30 min. After washing (PBS 3 x), the substrate (AEC substrate-Chromogen, Agilent Technologies, Dako, Cat: K346430–2, 2 drops) was dropped on the cells and incubated until viral infected cells were stained red, but not longer than 3 min. Reaction was stopped with washing in PBS (3 x) and wells were kept humid until photo documentation.

Statistical analysis

Statistical analysis was performed using Graphpad Prism 9. Significance was determined using mean rank comparison (for two groups Mann-Whitney test, for more than two groups Kruskal-Wallis test: comparing the mean rank of control column (no substance) with the mean rank of every other column in the graph). Significance for p-values is defined as: p > 0.5 (ns); p ≤ 0.05 (*); p ≤ 0.01 (**); p ≤ 0.001 (***). (GraphPad Software, San Diego, CA, USA).

Results

The GTE has been fully analysed and characterized with different analytical methods including identity (HPLC, HPTLC; HPTLC data not shown), purity, molecular weight distribution (SEC) and assay of catechins and polyphenols (HPLC and photometry; photometry data not shown).
shown). Concerning contaminations, the extract has been found to be in full compliance with the European regulations on pesticides (reg. (EC) No. 396/2005), polycyclic aromatic hydrocarbons, pyrrolizidine alkaloids and heavy metals (reg. (EC) No. 1881/2006), and the specification of residual solvents and microbiological purity of the European Pharmacopoeia (Ph. Eur. Monograph 10.0/5.4 and 10.0/5.1.8. B) (data not shown).

By HPLC analyses the eight catechins (-)-gallocatechin (peak no. 1), (-)-epigallocatechin (2), (+)-catechin (3), EGCG (5), (-)-epicatechin (6), (-)-gallocatechin-3-O-gallate (7), (-)-epicatechin-3-O-gallate (9) and (-)-epigallocatechin-3-O-(3′-O-methyl)-gallate (10) could be identified by retention time and DAD-spectra (cf. Fig. 1). The UV-spectrum of peak no. 8 indicates that this substance is a catechin derivative. According to UHPLC-HRMS analysis it could be (-)-epigallocatechin-3-O-methyl-gallate (data not shown). The most dominant catechins were EGCG (5) and (-)-epicatechin-3-O-gallate (9). Caffeine (peak no. 4 in Fig. 1) is present in GTE in a concentration of less than 1% (data not shown). By the HPLC assay, the total content of catechins, calculated as catechin, of 76 g/100 g was determined. SEC analysis in combination with molecular weight calibration revealed that also higher polymeric substances with a molecular weight of at least 9200 g/mol (peak no. 11 in Fig. 2) e.g. polymers of catechins or flavonoids, are present in GTE (Fig. 2). Due to their high molecular weight, these catechin derivatives are not targeted by the HPLC assay. SEC analysis also showed a big fraction of catechins and low molecular catechin derivatives (peak no. 12). HPLC analysis of the sGTE led to similar chromatograms (data not shown), indicating that the catechins of the extract were present unchanged in the spray.

**Cell viability / Metabolic Activity Assay**

**VeroE6 cells tolerate sGTE at certain concentrations and incubation periods**

In order to investigate possible effects on cell viability or metabolic inferences from application of sGTE to VeroE6 cells *in vitro*, a resazurin-based assay, with various concentrations of sGTE, was performed. Metabolic activity (MA) of sGTE treated and untreated VeroE6 cells was determined via calculating the slopes (k) from relative fluorescent units (RFU) of linear regression curves measured at 485/590 nm and comparing them to each other. Two set-ups were tested: Fig. 3 A displays the incubation with sGTE for 24 h followed by a washing step and subsequent determination of the RFU. The calculation of the relative metabolic activity [RMA % = (treated/untreated) x 100] resulted in an 88% growth of the untreated control (Supplementary Figure 1 A). In Fig. 3 B the MA for 1 h substance treatment, subsequent washing and additional 24 h incubation in MEM 2% is shown. In this experimental design, VeroE6 cells tolerate higher concentrations of sGTE, up to a concentration of 1:450 without evident cytotoxic effects or morphological changes; the RMA is approximately 70% for this sGTE concentration (Supplementary Figure 1 B).

Based on the outcome of the MA assays, the virus neutralization experiments were designed. The concentration of 1:4500 sGTE in MEM 2% was used when sGTE was present during infection and incubation (Fig. 4 A and B). When cells or virus were either preincubated with sGTE or treated only during the infection period of 1 h, a 1:450 dilution of sGTE in MEM 2% was applied (Figure 5).

**Virus neutralization assay**

**sGTE lowers viral replication**

To assess the antiviral effect of sGTE, VeroE6 cells were treated with sGTE and infected with a calculated MOI = 0.002 of SARS-CoV-2 for 1 h, followed by washing and subsequent incubation with sGTE. The positive control was treated the same w/o sGTE. qRT-PCR from supernatants showed that sGTE reduced the viral load significantly, compared to the no substance control (Fig. 4 B + C). As the different calculated virus input was used in two independent experimental series (Fig. 4 A), the reduction of viral copies when compared to the untreated positive control was in experiment of Fig. 4 B 3.5E+03-fold, and in the experiment of Fig. 4 C, 6.0E+02-fold. Consequently, a 600- to 3500-fold reduction of viral particles was observed, depending on the actual virus input per well indicating a dose-dependent activity.

To clarify that the reduction of virus particles relies on GTE and not on the excipients, experiments with various formulations were performed (Supplementary Figure 2) and the hypothesis of GTE as the trigger of virus reduction could be confirmed.

Next, we investigated whether sGTE inhibited viral replication by inactivating the virus, blocking infection of cells, or by reducing viral replication in infected cells. To identify the substance target, an experimental set-up covering various protocols was established (Table 2).

**Virus neutralization assay and immunohistochemistry**

**The efficiency of inhibition on viral infection depends on interaction of virus and sGTE**

Table 2 gives an overview of tested protocols to identify sGTE target of virus infection inhibition. The protocols include different parameters of sGTE application, either on cells, virus or both targets in parallel. Pre-treatment of cells with sGTE i) had the lowest effect compared to the other tested protocols (Fig. 5 A), the viral copies were reduced to a 37–fold factor compared to the no substance positive control. 6.7E+04 viral copies were detected via qRT-PCR 24 h post infection, 2.53E+06 viral copies via qRT-PCR 24 h post infection. In this experimental design, VeroE6 cells tolerate higher concentrations of sGTE, up to a concentration of 1:450 without evident cytotoxic effects or morphological changes; the RMA is approximately 70% for this sGTE concentration (Supplementary Figure 1 B).

Based on the outcome of the MA assays, the virus neutralization experiments were designed. The concentration of 1:4500 sGTE in MEM 2% was used when sGTE was present during infection and incubation (Fig. 4 A and B). When cells or virus were either preincubated with sGTE or treated only during the infection period of 1 h, a 1:450 dilution of sGTE in MEM 2% was applied (Figure 5).

**Virus neutralization assay**

**sGTE lowers viral replication**

To assess the antiviral effect of sGTE, VeroE6 cells were treated with sGTE and infected with a calculated MOI = 0.002 of SARS-CoV-2 for 1 h, followed by washing and subsequent incubation with sGTE. The positive control was treated the same w/o sGTE. qRT-PCR from supernatants showed that sGTE reduced the viral load significantly, compared to the no substance control (Fig. 4 B + C). As the different calculated virus input was used in two independent experimental series (Fig. 4 A), the reduction of viral copies when compared to the untreated positive control was in experiment of Fig. 4 B 3.5E+03-fold, and in the experiment of Fig. 4 C, 6.0E+02-fold. Consequently, a 600- to 3500-fold reduction of viral particles was observed, depending on the actual virus input per well indicating a dose-dependent activity.

To clarify that the reduction of virus particles relies on GTE and not on the excipients, experiments with various formulations were performed (Supplementary Figure 2) and the hypothesis of GTE as the trigger of virus reduction could be confirmed.

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particles in the no substance control (mean of 6 replicates). ii) preincubation of the SARS-CoV-2 virus with sGTE lowered the amount of virus copies for approximately 1400-fold to a mean virus copy number of \(5.67 \times 10^4\), compared to \(8.03 \times 10^7\) in the positive control (Fig. 5 B). iv) The presence of sGTE during 1 h infection reduced the viral copy numbers in a more effective (2780-fold) but comparable manner to ii) (Fig. 5 B). When iv) sGTE was present during 1 h infection a mean of \(2.88 \times 10^4\) viral copies were found after 24 h incubation. The parameter iii) pre virus infection led to the most remarkable reduction of viral copies to approximately \(1.18 \times 10^3\) detectable copies. This yields a 63,500-fold reduction as compared to the no substance control (Fig. 5 B). The obtained \(c_q\) values and calculated copy numbers from the qRT-PCR from this experiment, were validated using an additional read-out method for the same experiment. IHC specific staining for SARS-CoV-2 for all parameters displayed in the graph of Fig. 5 B are shown in Fig. 5 C. Infected cells are red. The results indicate that virus neutralizing activity of sGTE is mainly related to inactivation of the virus and not by reducing virus replication in infected cells.

**sGTE has virus neutralizing activity for the SARS-CoV-2 beta and delta variants**

In addition to the original SARS-CoV-2 isolate (Wuhan strain), the virus neutralizing activity of sGTE was investigated with the beta-variant. When sGTE was present during 1 h infection and 24 h incubation at a concentration of 1:4500 sGTE in MEM 2% the amount of virus copies after 24 h incubation was \(1.2 \times 10^3\) (Fig. 6 A). When sGTE was present only during infection at a concentration of 1:450 sGTE \(4.7 \times 10^2\) viral copies were measured after 24 h incubation (without sGTE) (Fig. 6 A). Consequently, a mean fold-change reduction of 6200 and 14,000, respectively, compared to the no substance positive control (\(8.04 \times 10^6\) viral copies) was calculated. For the delta-variant, preincubation of virus with 1:450 sGTE revealed a mean of \(1.27 \times 10^2\) viral copies (Fig. 6 B), a
590-fold decrease compared to the no substance control (7.47E+04 viral copies) infected with the delta variant. When sGTE was present only during 1 h infection (concentration 1:450) 1.24E+02 viral copies were detected after 24 h incubation. When virus was preincubated with 1:450 sGTE and additionally present on the cells during 1 h infection, 8.87E+01 copies were detected in the supernatant after 24 h incubation (Fig. 6 B). An 842-fold decrease of viral copies was measured for this parameter compared to the no substance control infected with the delta-variant.

In an additional small-scale assay, sGTE effectiveness to lower the viral load was tested in parallel to the neutralizing activity of antibodies derived from recovered patients (Data shown in Supplementary Figure 3). Patient derived serum (r-serum 20: recovered in 2020; not vaccinated and r-serum 21: recovered in 2021; vaccinated twice, confirmed infection with delta-variant) was preincubated with Wuhan and delta-strains. The experiment, performed in the same way as and in parallel to sGTE according to iii) pre virus + infection protocol (Table 2), revealed a dose-dependent neutralizing activity. By comparing the reduction of viral copies with 2 µl and 10 µl of serum with different strains (Wuhan and delta, MOI 0.002 each), a higher neutralizing effect was observed from patients recovered in 2021. A lower amount of serum was necessary to gain the same neutralization effect as for patients recovered in 2020, relative to the corresponding no substance / no serum control of the virus strains. This experiment was performed to visualize the effective range of sGTE in a direct comparison to the effect of neutralizing antibodies, resulting in the observation of a similar trend of decrease in detected viral particles.

**sGTE throat swab experiments**

In order to confirm that the concentrations tested in vitro are also relevant for the in vivo situation, we determined the qualitative and quantitative tannin profile present on the oral mucosa after application of the sGTE spray. Six healthy adult volunteers (4 females, 2 males, aged 28 - 65 years) have been applying two puffs of the preparation into the pharynx at 0, 30, 60, 90, and 120 min. Pharyngeal swabs were taken before and immediately after applying the first sGTE dose, and right before the application of the next sGTE dose. Two further swabs were taken 30 and 60 min. after the last sGTE dose.

**LC-MS/MS analysis of throat swabs taken after pharyngeal sGTE application shows consistent presence of major catechins throughout the experiment**

In a 1:1800 dilution of sGTE used for the experiment, the main
Fig. 5. Different sGTE treatment protocols from two independent experiments. A) Produced viral copy numbers after pretreatment of VerE6 cells (pre cells) with sGTE in MEM 2% and 24 h incubation compared to the positive control (no substance). B) The columns show the mean ± SD of n ≥ 6 replicates per tested condition; including single data points. Assay readout (A, B): qRT-PCR with SARS-CoV-2 specific primers and probe; viral copy numbers were calculated based on a serial diluted standard curve. The graphs were analysed for significance with an (A) Mann-Whitney test (not significant) and (B) Kruskal-Wallis test (p = 0.0008 (***) using Graphpad Prism 9. C) SARS-CoV-2 specific immunohistochemical staining (IHC) of cells treated with different infection protocols (2nd readout from experiment shown in B). Virus infected cells appeared red. The protocols ii) pre virus and iv) infection showed single infected cells in a confluent cellular monolayer. For iii) pre virus + infection, no infected cells were detected in the well. The positive control v) no substance, showed a dominant red cell staining.
signals in the HESI negative mode could be assigned to catechins on the basis of their m/z values and MS/MS fragmentation patterns, and by comparing their elution order with that described by Xin et al. (2018). When the throat swabs were analysed with the same method, the major catechins detected in the sGTE preparation were detectable in all swab extracts except for the control swab taken prior to sGTE application (Fig. 7). In the swab taken at 177 min, i.e. 1 h after the last spray application (Fig. 7 - line G), stronger changes in the LC-MS/MS profile were observed; in particular, the levels of EGCG (peak no. 5) decreased. Nevertheless, all major catechins were still detectable in these samples. This indicates that the catechin fingerprint of the preparation is not severely changed when applied in regular intervals to the pharyngeal region.

Pharmacologically relevant EGCG levels are detectable in the throat swabs

With the optimized gradient, the major green tea catechin EGCG was detected at 10.5 min and was baseline-separated from the minor catechins, and the internal standard eluted at 19.5 min (Supplementary Figure 4). A linear correlation was established over a concentration range from 0.09 to 186 μg/ml (y = 0.027x-0.1041, R² = 0.9959). The method provided excellent intra- and inter-day precision: concerning intraday variability, relative standard deviation (RSD) of the determined EGCG concentration was 0.48%, and concerning inter-day variability, RSD was 2.59%, based on the average of measurements performed in

Fig. 7. HESI negative mode full MS chromatograms (scan range m/z 100–1000) of sGTE at a dilution of 1:1800 and of throat swabs taken at 1 min. (A), 27 min. (B), 57 min. (C), 87 min. (D), 117 min. (E), 147 min. (F) and 177 min. (G) of the experiment; peak assignment according to m/z values and elution order described by Xin et al. (2018): 1 = gallocatechin (m/z 305), 2 = epigallocatechin (m/z 305), 3 = catechin (m/z 289), 4 = epicatechin (m/z 289), 5 = epigallocatechin-3-O-gallate (m/z 457), 6 = gallocatechin gallate (m/z 457), 7 = (epi)gallocatechin-methyl-O-gallate (m/z 471), 8 = (epi)catechin gallate (m/z 441), 9 = epiafzelechin gallate (m/z 425).
triplicates on three different days. LOQ (signal-to-noise ratio >5) was found to be around 0.47 µg/ml, and LOD (signal-to-noise-ratio >3) around 0.23 µg/ml. Accuracy was found to range between 95.6 and 104.7% of the expected concentration (Table 3). The recovery of the method used for extracting the throat swabs ranged between 79.8 and 90.9%, indicating that a minor proportion of EGCg was obviously retained on the swabs (Table 3).

When the throat swab extracts were analysed with this optimized HPLC-DAD method, EGCg was undetectable in the control samples taken prior to sGTE application, but detectable in all other samples except for the 27 min samples of volunteers 2 and 4 (Supplementary Table 1). In order to allow an estimation of the EGCg concentration present in the saliva taken from the pharyngeal region by the throat swabs, probands were asked to perform three further throat swabs without sGTE application and to weigh the swabs before and after deduction. From the mean resulting weights in mg, the mean saliva volume in µl was calculated (the density of saliva is 1.01–1.02 g/ml). Based on that, the approximate concentration present in saliva was calculated by normalizing the EGCg amounts detected in the throat swabs to the average saliva volumes (Supplementary Table 2).

The highest and most varying EGCg concentrations were detected in the 1 min samples that were taken directly after applying the first sGTE dose (Fig. 8). The lowest levels were measured in the samples of the second swab that was taken 27 min after the first sGTE dose. At the following time points, when the swabs were taken briefly before applying the subsequent sGTE dose, detected median EGCg amounts consistently ranged between 344 and 407 µg/ml saliva and slightly decreased in the last swab that was taken 1 h after application of the last sGTE dose (median concentration 259 µg/ml).

In order to be able to correlate the approximate EGCg concentrations present in saliva in the throat swab experiment with the concentration present in the cellular in vitro studies on antiviral activity of the preparation, the EGCg concentration of sGTE at a dilution of 1:450, i.e. the higher concentration used in the cellular assays, was determined. EGCg was found to be present in this dilution at a concentration of 102 µg/ml; thus, the EGCg concentration of the 1:4500 dilution can be assumed to be around 10.2 µg/ml.

Therefore, the approximate EGCg concentrations present in saliva throughout the throat swab experiment were in the range or above the EGCg concentrations reached with sGTE 1:450 dilution in the cellular assays on antiviral activity (Fig. 8, Supplementary table 2).

### Discussion

Regarding the impact of the recent COVID-19 pandemic, it is mandatory to develop different types of effective treatments and medications against the virus. Certainly, vaccines play a central role in fighting the COVID-19 pandemic and demonstrated very good efficacy to prevent severe disease, but additional preventive and supportive therapeutic approaches are needed. This is of particular relevance to cope with emerging new variants which may differently impact on vaccines and other interventions based on different mode of actions (Planas et al., 2021). New virus variants develop over the time during the viral evolutionary adaptation to the human as well as environmental factors (Jie et al., 2019). Further, human to human transmission is not efficiently avoided until a certain percentage of the population either has the chance or is willing to be vaccinated or developed immunity by overcoming infection. From the research point of view, providing new or repurposing approved drugs on a different target is a very time-consuming process as patient safety while application must be an overall aim and this requires laborious pre-clinical and clinical evaluation before a product can be approved and put on the market. This opens application possibilities for products based on plant extracts and natural sources, which have commonly known broad antiviral or antibiotic range and are well tolerated without severe side effects (Xian et al., 2020), e.g. green tea (Mhatre et al., 2021a).

The relative metabolic activities determined for the sGTE concentrations tested excluded major effects on cell viability or metabolic alterations, which may non-specifically reduce virus replication and could pretend virus neutralizing activity. The findings of the Resazurin MA assay were also in line with the well-preserved morphology of the cells showing no evidence of cytotoxic effects (Fig. 5 C). The VeroE6 cell line was chosen for the in vitro assays, because they have the highest susceptibility for viral infections and are an established model for virus propagation and substance testing (Rosa et al., 2021). It is known that the mode of action of GTE is mainly relying on virus - substance interaction (Mhatre et al., 2021b; Joseph et al., 2021). So, there was no need to use human cells.

The different designs of the virus neutralizing assay suggest that sGTE directly interacts with the virus, since preincubation of the virus and absence of the substance during infection or incubation periods reduced the viral particles effectiveness to infect cells. The virus neutralizing activity found in experiments in which sGTE was also present during the 24 h incubation period could be explained by inhibition of virus production by infected cells and prevention of infection of other cells, which already occurs within 24 h. However, additional interaction with virus replication cannot be excluded.

At least in silico, epicatechin-3,5-di-O-gallate, epigallocatechin-3,5-di-O-gallate, and epigallocatechin-3,4-di-O-gallate showed better interaction with RNA-dependent RNA polymerase than antiviral drugs Remdesivir and Favipiravir (Bhardwaj et al., 2021). It has been shown in vitro that EGCG treatment decreased 3CL-protease activity of HCoV-OC43 and HCoV-229E, as well as HCoV-OC43-induced cytotoxicity (Liu et al., 2021b, Jang et al., 2021). Other studies have shown that EGCG effectively blocks infection of SARS-CoV-2 and new variants by inhibiting spike binding to ACE2 receptor (Joseph et al., 2021; Liu et al., 2021a) and EGCG has been suggested for prevention COVID-19 (Tsvetkov et al., 2021). It was also shown, that EGCG blocks not only the entry of SARS-CoV-2, but also MERS- and SARS-CoV pseudo-typed lentiviral vectors and inhibited virus infections in vitro (Hens et al., 2021). However, also other constituents may be relevant. Recently, it was shown that (-)-gallocatechin gallate inhibits SARS-CoV-2 replication by disrupting the liquid phase condensation of its nucleocapsid protein (Zhao et al., 2021). According to our in vitro results, sGTE has a high virus reducing potential and a virus-variant independent mode of action. These findings are in line with previous investigations showing that green tea extract has antiviral properties (Liu et al., 2021a; Takeda et al., 2021).

Our results have shown, that green tea polyphenols are consistently detectable on the human throat mucosa for more than 3 h, and that the formulations is well tolerated in vivo. This leads to the assumption that the formulation might reduce the probability of a viral infection when periodically applied to mouth and throat preventively or in situations, when enhanced prophylactic protection is needed.

### Conclusion

The findings of this study suggest that sGTE has a strong neutralizing activity on SARS-CoV-2 independent from the strain (Wuhan strain, ...
As also shown in previous studies, it is most likely that the sGTE antiviral activity depends on catechins like EGCG and other polyphenols. The type of interaction of sGTE with surface proteins seems to be direct but non-specific concerning the viral surface protein structures and resembles the general non-specific activity of polyphenols (Chung et al., 1998). Studies in humans have shown that the concentrations of green tea tannins in the pharyngeal mucosa after spraying the sGTE preparation are higher than the effective dose found in the in vitro studies with SARS-CoV-2, even 1 h after the last application. Therefore, sGTE may be valuable for prevention of corresponding viral infections when periodically applied to mouth and throat, and should be further investigated in clinical studies as suggested by Mahmoodi et al. (2021).

**Authorship statement**

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in Phytomedicine Journal.

**Funding**

This study has been funded by Phytovisions GmbH & Co. KG, (Garmisch-Partenkirchen, Germany).

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**Declaration of Competing Interest**

The financial support for this study from Phytovisions GmbH & Co. KG, Germany, is disclosed. Gerolf Tittel is the founder and owner of Phytovisions. All other authors report no declarations of interest.

**Acknowledgments**

The authors would like to thank the lab team of Prof. Zatloukal, especially Stephanie Freydl for performing the qRT-PCR analyses, as well as Melina Hardt, Esther Föderl-Höbenreich and Julia Rieger for assistance and accompaniment at the BSL-3 laboratory. We also want to thank the Biobank of Medical University Graz for providing Covid-19 convalescent serum.

**Supplementary materials**

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2022.153970.

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