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Insights into the direct anti-influenza virus mode of action of *Rhodiola rosea*

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ABSTRACT

Background: The anti-influenza A virus activities and contents of previously isolated most active flavonoids (rhodiosin and tricin) from a standardized hydro-ethanolic *R. rosea* root and rhizome extract (SHR-5®), did not fully explain the efficacy of SHR-5®. Moreover, the mode of antiviral action of SHR-5® is unknown.

Purpose: To determine the anti-influenza viral principle of SHR-5® we evaluated i) the combined anti-influenza virus effect of rhodiosin and tricin, ii) the impact of its tannin-enriched fraction (TE), iii) its antiviral spectrum and mode of action, and iv) its propensity for resistance development *in vitro*.

Methods: The combined anti-influenza virus effect of rhodiosin and tricin and the impact of TE were investigated with cytopathic effect (CPE)-inhibition assays in MDCK cells. A tannin-depleted fraction (TD) and TE were prepared by polyamide column chromatography and dereplicated by LC-MS. Plaque-reduction assays provided insights into the anti-influenza virus profile, the mode of action, and the propensity for resistance development of SHR-5®.

Results: Our results i) did not reveal synergistic anti-influenza A virus effects of rhodiosin and tricin, but ii) proved a strong impact of TE mainly composed of prodelphinidin gallate oligomers. iii) TE inhibited the plaque-production of influenza virus A(H1N1)pdm09, A(H3N2), and B (Victoria and Yamagata) isolates (including isolates resistant to neuraminidase and/or M2 ion channel inhibitors) with 50% inhibitory concentration values between 0.12 - 0.53 µg/ml similar to SHR-5®. Mechanistic studies proved a virucidal activity, inhibition of viral adsorption, viral neuraminidase activity, and virus spread by SHR-5® and TE. iv) No resistance development was observed *in vitro*.

Conclusion: For the first time a comprehensive analysis of the anti-influenza virus profile of a hydro-ethanolic *R. rosea* extract (SHR-5®) was assessed *in vitro*. The results demonstrating broad-spectrum multiple direct anti-influenza virus activities, and a lack of resistance development to SHR-5® together with its known augmentation of host defense, support its potential role as an adaptogen against influenza virus infection.

Abbreviations

CPE	cytopathic effect
EGCG	(-)-epigallocatechin gallate
IC_{50}	50% inhibitory concentration
MDCK cells	Madin Darby canine kidney cells
SHR-5®	standardized *R. rosea* root and rhizome extract
TD	tannin-depleted fraction of SHR-5®
TE	tannin-enriched fraction of SHR-5®
TCID50	tissue culture infection dose 50%
PFU	plaque forming unit
UHPLC-HRMS—	Ultra performance liquid chromatography coupled to high resolution mass spectrometry

Introduction

Influenza viruses of types A (subtypes A(H1N1) pdm09 and A (H3N2)) and B (genetic lineages Victoria and Yamagata) represent a significant health burden by causing mild to severe acute respiratory infections. Influenza causes an annual death toll of 290,000–650,000 people worldwide during seasonal epidemics (WHO, 2021). During influenza pandemics, morbidity and mortality increase significantly

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(Short et al., 2018).

Vaccines and drugs targeting the viral neuraminidase (zanamivir, oseltamivir, peramivir, and laninamivir) and polymerase (favipiravir and baloxavir marboxil) allow the prevention and/or treatment of influenza. Due to resistance of circulating viruses to M2 ion channel blockers (amantadine and rimantadine), the use of these drugs is not recommended today (Duwe et al., 2021). This narrow influenza drug arsenal together with the permanent risk of drug resistance development urgently call for new agents to combat influenza.

Medicinal plants and fungi constitute a rich reservoir for inhibitors of influenza virus replication (Grienke et al., 2012; 2018; Langeder et al., 2020a). For centuries, the dried roots and rhizomes of *Rhodiola rosea* L. (syn. *Sedum roseum* (L.) Scop., Crassulaceae), also called "roseroot", "golden root" or "Arctic root", have been used for its adaptogenic effects, and are on the market as herbal medicinal products and food supplements in many parts of the world (Panossian, Wikman and Sarris, 2010b). According to the guidelines of the European Medicines Agency's Committee on Herbal Medicinal Products, roseroot is traditionally used "for temporary relief of symptoms of stress, such as fatigue and sensation of weakness" (EMA/HMPC/232,091/2011). The multiple activities of adaptogens and their constituents make them also interesting for prophylaxis and treatment of viral respiratory infections (Brendler et al., 2021; Panossian and Brendler, 2020). Evidence for the application of roseroot extracts to target respiratory infections is scarce: it is reported as one of the 13 herbs of Lianhuaqingwen Capsule, a traditional Chinese medicine formula which is used to treat respiratory tract infections in China, also showing anti-influenza virus activity *in vitro* (Ding et al., 2017) and *in vivo* (Gao et al., 2020). Components of Lianhuaqingwen Capsule might also contribute in inhibiting a SARS-CoV-2 infection (Chen et al., 2021). Flavonoids isolated from roseroot have been reported to inhibit the activity of neuraminidases and replication of influenza A(H1N1) and A(H9N2) viruses (Jeong et al., 2009).

Recently, we confirmed a distinct anti-influenza virus activity of the standardized hydro-ethanolic extract of *R. rosea*, SHR-5®, *in vitro*. Among the isolated metabolites were compounds from the structural classes of phenylethanoids, phenylpropanoids, cyanogenic and monoterpene glycosides, and flavonoids. It was primarily the latter compound class, consisting of tricin, tricin-5-O-β-D-glucopyranoside, and rhodiosin, which exhibited an anti-influenza A virus activity (Langeder et al., 2020b). However, the activities of the isolated minor constituents targeting influenza virus did not fully explain the strong inhibitory effect of SHR-5®.

Thus, the aim of the present study was to identify the anti-influenza viral principle of SHR-5®. For this purpose, we evaluated a potential synergistic antiviral effect caused by the previously identified most bioactive flavonoids tricin and rhodiosin, determined the tannin content and explored the impact of a tannin-depleted (TD) and a tannin-enriched fraction (TE) of SHR-5®. Since the latter fraction showed a promising anti-influenza virus activity, we aimed to determine its composition and anti-influenza profile using five currently circulating type A and B influenza virus strains with different drug susceptibilities. Assuming a direct antiviral activity of SHR-5® and its main constituents, we sought to illuminate the specific stages in the viral replication cycle targeted by SHR-5® and TE and the propensity of SHR-5® for resistance development.

Material and methods

Plant material, isolated compounds, and reagents

SHR-5® (batch no. 1,521,229), a hydro-ethanolic dry extract of *R. rosea* root and rhizome prepared with 70% ethanol (first extraction solvent) and water (second extraction solvent) with a drug-extract-ratio of 2.5 - 5:1 in accordance to HMPC guidelines (EMA/HMPC/232,100/2011), was obtained from M. Jafari in 2018, provided by the Swedish Herbal Institute. SHR-5® is an extract standardized for the content of salidroside (1.0 - 3.0%) and rosavin (1.5 - 3.0%). A voucher specimen (JR-20,180,904-A1) is deposited at the Department of Pharmaceutical Sciences, Division of Pharmacognosy, University of Vienna, Austria. A UPLC fingerprint of the extract was published recently (Langeder et al., 2020b). Rhodiosin and tricin were isolated from SHR-5® with purities, according to the UPLC-ELSD signal, of 97% and 95%, respectively (Langeder et al., 2020b).

Oseltamivir carboxylate GS4071 (oseltamivir; Roche Pharma AG, Basel, Switzerland), zanamivir (GlaxoSmithKline, Brentford, UK), and amantadine (SIGMA-Aldrich Chemie GmbH, Steinheim, Germany) served as reference compounds. Stock solutions were prepared in DMSO (SHR-5®: 10 mg/ml; rhodiosin and tricin: 10 mM) or water (oseltamivir and zanamivir: 10 mM).

Epicatechin gallate, catechin, epicatechin and corilagin (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) served as analytical standards. Stock solutions were prepared in methanol (1 mg/ml).

All solvents were purchased from VWR International GmbH (Fontenay-sous-Bois; France). Acetonitrile for UPLC analysis was of chromatographic grade.

Preparation of tannin-depleted and tannin-enriched fractions and their UHPLC-HRMS qualitative analysis

For tannin enrichment 0.5 g of SHR-5® were subjected to column chromatography using polyamide (Carl Roth GmbH & Co. KG) as stationary phase (~4 g, 40 × 0.5 cm). Before applying the sample, the polyamide gel was washed with 40 ml water, followed by 30 ml methanol. Stepwise elution was performed using 94 ml of methanol, 60 ml of a mixture of methanol-acetone-water (80:16:4), 120 ml of a mixture of acetone-water (70:30) and finally 30 ml water to yield the fractions TD (259.3 mg) and TE (23.4 mg).

UHPLC-HRMS— analysis of TE was performed on an EXIONLC AD SYSTEM (AB Sciex, Darmstadt, Germany) system coupled to a turbo ion source ESI X500 QTOF mass spectrometer (AB Sciex). TE was separated on a Waters CSH C_{18}— column (2.1 × 100 mm, 1.7 µm) within 30 min. Solvent A: Water-acetic acid-formic acid (99.9:0.05:0.05), Solvent B: Acetonitrile-acetic acid-formic acid (99.9:0.05:0.05). The following gradient was applied: 0–5 min: 0% B, 5–26 min: 95% B, 26–28 min 95% B, 28–30 min 0% B. 5 µl of dissolved sample (methanol-water 25:75) were injected. MS parameters: 500 °C heater temperature, ion source gas 1 set to 30 psi, ion source gas 2 set to 30 psi, curtain gas set to 45 psi, −4.5 KV/+5.0 KV spray voltages were applied to achieve negative/positive ion mode ionization.

Cells

Cell-culture assays were performed with Madin Darby canine kidney (MDCK) cells (Friedrich Löffler Institute, Riems, Germany), with Eagle's minimal essential medium supplemented with 2 µg/ml trypsin, 2 mM L-glutamine, and 1% nonessential amino acids.

Human erythrocytes (blood group O; department transfusion medicine, Jena University Hospital, Germany) were stored at 4 °C and washed three times in phosphate-buffered saline without Ca^{2+} and Mg^{2+} before use.

Viruses

Influenza viruses A/HK/1/68 (Schaper and Brümmer GmbH & Co. KG, Salzgitter, Germany), A/Jena/8178/09 (Schmidtke et al., 2001; Walther et al., 2016), A/BLN/11/2019, A/BLN/7/2019, A/BLN/36/2019, B/NRW/33/2018, and B/SN/59/2018 (isolated from nasal swabs provided by physicians participating in the German virologic influenza sentinel system at the Robert Koch-Institute, Berlin, Germany (Duwe and Schweiger 2008)) were propagated and titrated in MDCK cells. Aliquots were stored at −80 °C.

Antiviral susceptibility testing of influenza virus isolates

Susceptibility of influenza viruses to neuraminidase inhibitors was determined by analyzing their 50% inhibitory concentration (IC_{50}) for enzymatic activity of neuraminidase in a fluorescence-based enzyme inhibition assay using 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (Munana; Biosynth AG, Staad, Switzerland) as previously described (Duwe and Schweiger, 2008; Rath et al., 2017).

Pyrosequencing analysis was used for detecting molecular resistance markers of neuraminidase and the M2 ion channel (Duwe et al., 2011).

Combination studies with tricin and rhodiosin

Potential additive or synergistic effects between tricin and rhodiosin were investigated in two-day-old confluent monolayers of MDCK cells. We added 50 µl of test medium (mock-treatment of cell control and virus controls; six respective replicates), single or combined half-log dilutions of the reference compound amantadine, tricin and/or rhodiosin in test medium to the cell monolayers. Each combination experiment included 0, 0.316, 1, 3.16, 10, 31.6 µM of each inhibitor alone and 36 different combinations thereof. Immediately thereafter, we inoculated influenza virus A/HK/1/68 (multiplicity of infection: 0.001 $TCID_{50}$/cell) in a volume of 50 µl of test medium. After incubating the plates at 37 °C for 48 h, cell monolayers were fixed, stained, and analyzed as published (Schmidtke et al., 2001).

Comparison of cytotoxicity and antiviral activity of SHR-5®, TD, and TE

Cytotoxic and CPE inhibitory effects of SHR-5®, TD and TE (10, 31.16, and 100 µg/ml; duplicates) against influenza virus A/HK/1/68 (multiplicity of infection: 0.001 $TCID_{50}$/cell) were analyzed in confluent MDCK cell monolayers as published (Schmidtke et al., 2001).

Plaque reduction assays

Plaque reduction assays were performed as described (Schmidtke et al., 2001) with following modifications: i) confluent MDCK cell monolayer in 12-well plates (Greiner AG, Kremsmünster Austria) were inoculated with 0.5 ml test medium (cell control) or virus suspension without (virus control; triplicate) or with serial half-log dilutions (0.031–10 µg/ml; duplicates) of SHR-5®, TD, or TE at 37 °C for 1 h, ii) after aspirating the inoculum 1 ml test medium containing 0.4% agar and the appropriate concentrations of SHR-5®, TD, and TE were added per well, iii) incubation proceeded at 37 °C for 48–72 h.

Time-of-addition assays

SHR-5®, TE (both 10 µg/ml), and zanamivir (1 µM; control) were added for different time periods and at different temperatures in plaque-reduction assays to analyze their inhibitory activity on different stages of the influenza virus A/HK/1/68 replication cycle as published (Walther et al., 2020). Briefly, MDCK cells or virus were treated for 1 h at 37 °C before infection, SHR-5®, TE, and zanamivir were added during virus adsorption (2 h at 4 °C), during penetration and uncoating (1 h at 37 °C), and during multiple replication cycles (48 h at 37 °C). After counting the plaques, the percentage of inhibition of plaque production was calculated.

Hemagglutination and neuraminidase inhibition assay with human erythrocytes

Four hemagglutination units of influenza virus A/Jena/8178/09 (25 µl) were incubated with eight serial half-logarithmic dilutions (25 µl; duplicates) of zanamivir, SHR-5®, TD, TE or phosphate-buffered saline (control) and a 1% erythrocyte suspension (50 µl) for two h at 4 °C (Walther et al., 2020). The lowest compound concentration inducing or inhibiting hemagglutination defined the minimum hemagglutination or the hemagglutination inhibitory concentration, respectively.

Further incubation at 37 °C for 24 h allowed the activation of viral neuraminidase and thus abrogation of hemagglutination. The lowest compound concentration that inhibits abrogation of hemagglutination represented the minimum neuraminidase inhibitory concentration (Walther et al., 2020).

Inhibition of virus spread

MDCK cells were infected in the absence of inhibitors with influenza virus A/HK/1/68 (multiplicity of infection 0.01 $TCID_{50}$/cell) for 1 h at 37 °C. Thereafter, non-adsorbed virus was removed by a washing step with test medium. Infected cells were mock-treated with test medium (virus control), treated with zanamivir (1 µM) or SHR-5® (10 g/ml) for 6 and 24 h. To visualize the number of virus-infected cells in the first replication cycle (6 h; infection control) and viral spread in the absence and presence of inhibitors after multiple replication cycles (24 h), viral nucleoprotein was immunocytochemically detected (Walther et al., 2016).

Resistance development studies

We incubated three independently prepared pools of influenza virus A/BLN/7/2019 with 3.16 µg/ml of SHR-5® in test medium for 1 h at 37 °C as described for other inhibitors (Braun et al., 2015). Then, duplicates of serial tenfold dilutions of all pools were added to confluent MDCK cell monolayers in the presence of SHR-5® and overlaid with agar containing SHR-5® (3.16 µg/ml). After 72 h incubation at 37 °C overall one plaque from each plate growing in the presence SHR-5® was picked. After four further plaque-to-plaque purifications in the presence SHR-5® (3.16 µg/ml) the sensitivity of the plaque-purified viruses to SHR-5® was compared with that of wild type virus in plaque reduction assays.

Statistical analysis

The raw data, mean values, standard deviation, and/or 95% confidence intervals (CI95) from the cytotoxicity, CPE inhibition, and plaque-reduction assays were analyzed using EXCEL 2016 software. IC_{50} values for inhibition of plaque production were calculated by using the Four Parameter Logistic (4PL) Curve Calculator (AAT Bioquest Inc (AAT Bioquest Inc et al., 2021). The numbers of experimental replicates are shown in the figure legends.

For statistical analysis of combination assay (Fig. S1), we applied a standard protocol and statistical evaluation program recommended for antiviral compounds (Prichard and Shipman, 1990). The percentage of CPE inhibition was subsequently transformed using the MacSynergy II software. All data points generated from the matrix of drug concentrations were used in this analysis. Triplicate data sets were used to perform statistical analysis with MacSynergy II software and determine the volume of synergy or antagonism at 99.9% confidence for the interpretation of the results by the program.

Based on the results of normality test, the One-way ANOVA or Kruskal-Wallis test (SigmaPlot 14.5) were applied to analyze (i) the anti-influenza virus activity of SHR-5®, TD, and TE (Fig. 2), (ii) the anti-influenza virus spectrum of SHR-5® and TD (Table S2), and (iii) the mode of action of SHR-5, TE, and the control compound zanamivir (Fig. 4).

Results and discussion

Evaluation of combinatory effects of rhodiosin and tricin

Focusing on small secondary metabolites from SHR-5®, we previously identified the flavonoids rhodiosin and tricin (Fig. 1) as strongest inhibitors of the influenza virus A/HK/1/68-induced CPE in MDCK cells

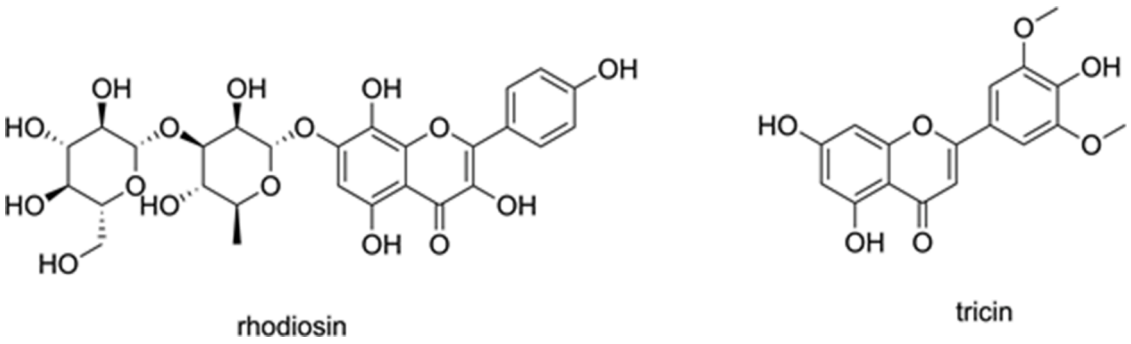

Fig. 1. Chemical structures of rhodiosin and tricin isolated from *R. rosea*.

(Langeder et al., 2020b). The analysis of SHR-5® with a previously published quantitative method using SFx technologies (Langeder and Grienke, 2021) resulted in the following contents of marker compounds: 1.6% salidroside and 3.1% rosavins (2.0% rosavin, 0.6% rosarin, 0.5% rosin). Moreover, the extract contains 0.6% tyrosol, 3.5% rosiridin and 0.3% tricin-5-O-β-D-glucopyranoside, which is a flavonoid closely related to the two investigated minor constituents tricin and rhodiosin. Considering the antiviral activities and the minor content of rhodiosin and tricin in SHR-5®, the strong anti-influenza virus activity of SHR-5® could not be fully explained.

To evaluate whether there is an amplification, potentiation, or synergism of tricin and rhodiosin, we performed further CPE inhibitory assays with 36 combinations of these compounds (concentrations from 0 to 31.6 μg/ml) and influenza virus A/HK/1/68 in MDCK cells. The obtained three-dimensional data plot resulted in a horizontal plane at 0% (Fig. S1) indicating a purely additive interaction of these two rose-root constituents. Therefore, the strong anti-influenza virus activity of SHR-5® is not based on a potentiation of tricin and rhodiosin.

Tannins mainly contribute to the anti-influenza virus effect of R. rosea extract

Various plant extracts are rich sources of tannins, which might impair influenza virus replication (Panossian and Brendler, 2020; Theisen and Muller, 2012). Determination of the tannin content was performed in duplicate according to the monograph in the European Pharmacopoeia 'Tannins in herbal drugs (2.8.14)' and revealed a total amount of 13.2% tannins in the rose root extract SHR-5®. To explore the impact of tannins on the strong anti-influenza virus activity of SHR-5® we depleted (TD) and enriched tannins (TE). SHR-5®, TD, and TE were well-tolerated in MDCK cells up to the maximal tested concentration of 100 μg/ml (Tab. S1). TD poorly inhibited the influenza virus A/HK/1/68-induced CPE, whereas SHR-5® and TE completely blocked the CPE induction as summarized in Tab. S1 and shown exemplarily for 10 μg/ml in Fig. 2.

Anti-influenza virus spectrum of SHR-5® and TE

The strong anti-influenza virus effect of both SHR-5® and TE was confirmed against a panel of current influenza virus isolates of types A and B. All three influenza A viruses possess the amino acid substitution S31N in matrix protein 2 (M2-S31N; Tab. S2) conferring M2 ion channel blocker resistance. Influenza B viruses are generally ion channel blocker-resistant (Wang et al., 2009). Amino acid substitution H275Y additionally confers oseltamivir resistance to influenza virus A/BLN/11/2019 neuraminidase (Tab. S2). The neuraminidases of the other influenza viruses are oseltamivir-susceptible. The dose-response analysis (Fig. 3A-C) and IC_{50} values (Tab. S2) demonstrate the inefficacy of TD and the efficacy of SHR-5® and TE even against antiviral-resistant influenza A and B viruses, thereby confirming the impact of condensed tannins for the anti-influenza virus activity of the SHR-5®. With the exception of SHR-5® activity against influenza virus A/BLN/11/2019 and B/NRW/33/2018, no statistically significant differences were identified when analyzing the 50% inhibitory activities of SHR-5® and TE against the panel of five current influenza virus isolates pairwise by applying On-Way ANOVA and Holm-Sidak method (Tab. S2).

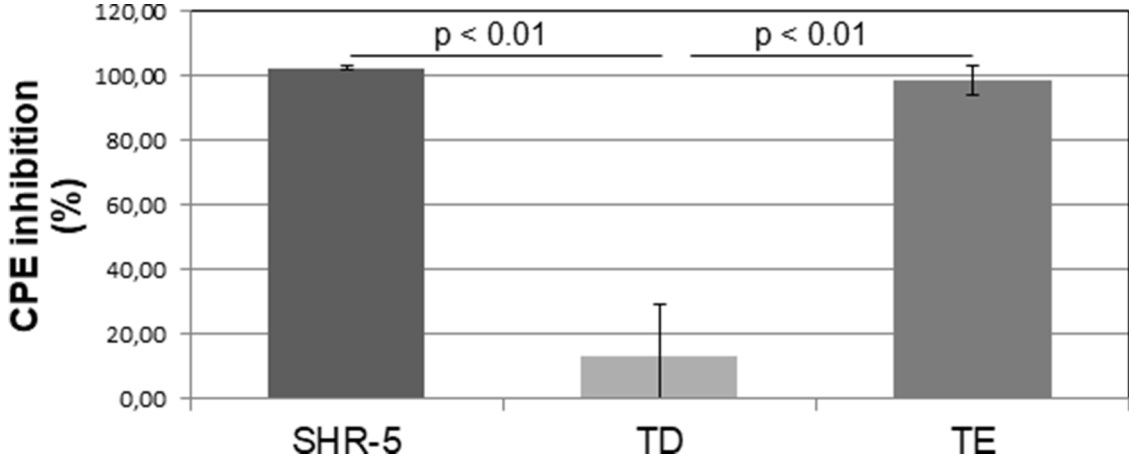

Fig. 2. SHR-5® and TE but not TD (c = 10 μg/ml, duplicates) inhibit the influenza virus A/HK/1/68-induced cytopathic effect (CPE) in MDCK cells. Two independent assays were performed to determine the mean percentages of CPE inhibition with standard deviations. One-way ANOVA and pairwise multiple comparison procedure (Holm-Sidak method) were applied in statistical analysis.

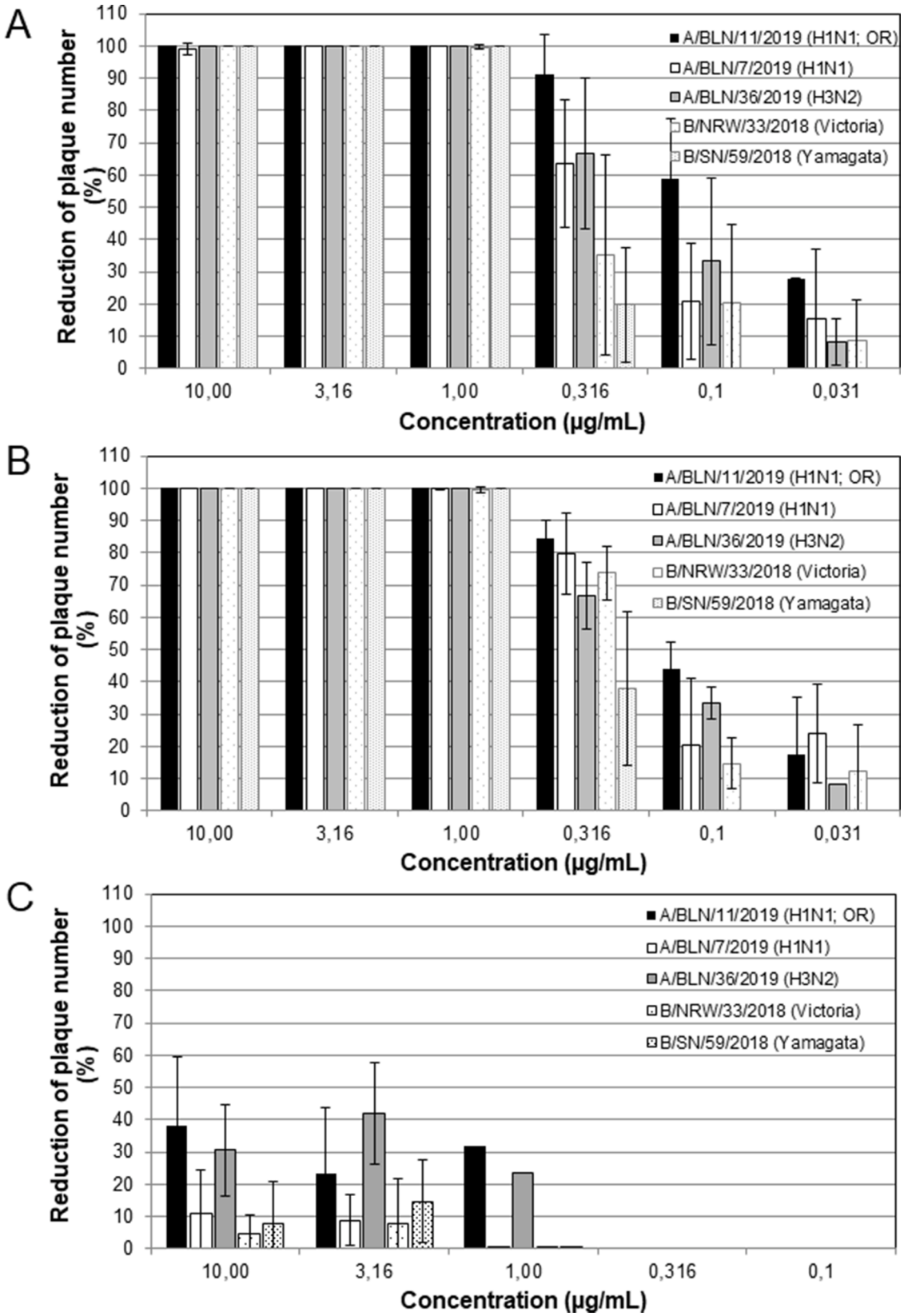

Fig. 3. SHR-5® (A) and TE (B) but not TD (C) exerted broad spectrum anti-influenza virus activity. Confluent MDCK cell monolayers were inoculated with virus suspensions without or with SHR-5®, TD, or TE (triplicates) at 37 °C for 1 h. After aspirating the inoculum, test medium containing 0.4% agar and the appropriate concentrations of SHR-5®, TD, and TE were added for 48–72 h. Three independent plaque reduction assays were performed to determine the mean percentages of reduction in plaque number with standard deviations.

TE contains proanthocyanidins

The Herbal Medicinal Products Committee (HMPC) recommends 67–70% ethanolic extraction for the medicinal use of roseroot. A 70% acetone extraction of three different *Rhodiola* species leads to a 30% content of condensed tannins (Yousef et al., 2006). Due to the strong anti-influenza activity of TE, UHPLC-MS/MS was performed to characterize its composition. TE is primarily composed of condensed tannins, among them catechins, epicatechins and epigallocatechin gallates of a different polymerization degree, but also ellagitannins and small monomeric phenolic compounds. Moreover, a —UHPLC-HRMS qualitative analysis was performed to confirm the presence of tannins in TE (Tab. S3).

In accordance to published investigations on the tannin-content of *R. rosea* (Gryszczyńska et al., 2012; Han et al., 2016), proanthocyanidins of the epigallocatechin gallate type were found to mainly constitute the bioactive TE. Interestingly, proanthocyanidins also represent the active principle of EPs® 7630 (Umckaloabo), an ethanolic root extract approved for the treatment of chronic bronchitis (Theisen et al., 2014).

SHR-5® and TE interact with the viral envelope and block virus adsorption

By using time-of-addition assays we investigated at which step of the viral life cycle SHR-5® and TE (both tested at 10 µg/ml) act against influenza virus A/HK/1/68. Zanamivir was included as control. Treatment of MDCK cells with SHR-5® and TE before infection had no effect. Treatment of virus with SHR-5® and TE before infection prevented plaque production (Fig. 4) indicating an interaction of SHR-5® and TE with the viral envelope that neutralizes infectivity (virucidal activity).

SHR-5® and TE also prevented plaque production when present during the 2 h of virus adsorption at 4 °C as well as during and after adsorption (Fig. 4). No reduction of plaque production occurred when SHR-5® and TE were added during penetration and uncoating or after adsorption of viruses (Fig. 4). As expected, zanamivir exerted its activity when added after virus adsorption. The results of statistical analysis are summarized in Table S4.

SHR-5® and TE block NA activity and virus spread

Hemagglutination inhibition assays were applied to investigate whether the proven interaction of SHR-5® with the influenza virus envelope affects the binding of viral hemagglutinin to its cellular receptors (sialic acids) and/or viral neuraminidase activity. As shown in Fig. 5A, no hemagglutination occurred in the absence of virus and inhibitors (Co1), and in the presence of zanamivir or TD. SHR-5® and TE caused a partial hemagglutination at concentrations equal or higher than 3.16 µg/ml indicating a binding of extract components to the erythrocyte membrane (Fig. 5A). However, virus-mediated hemagglutination (Co2 in Fig. 5A) and thus, the hemagglutinin-receptor binding was not inhibited. Interestingly, Lianhuaqingwen Capsule was recently reported to inhibit the binding between ACE2 and S protein SARS-CoV-2 (Chen et al., 2021).

The activated viral neuraminidase cleaved the link between hemagglutinin and receptor (Fig. 5B, Co2), thus abrogating the virus-mediated hemagglutination. TD did not affect the neuraminidase activity. Zanamivir, SHR-5®, and TE were active with minimal inhibitory concentrations of 0.03 µM, 0.30 µg/ml, and 0.17 µg/ml, respectively (Fig. 5B).

Inhibition of the neuraminidase activity prevents virus spread. This was confirmed for SHR-5® and zanamivir (positive control). Both were added after infecting MDCK cells with a low virus dose of influenza virus A/HK/1/68 ensuring the infection of very few cells in the first replication cycle at 6 h p.i. (red dots in Fig. 6). The viral nucleoprotein was detected by immunocytochemical staining in nearly all cells of the untreated virus control (red staining, 24 h after infection). In contrast, zanamivir and SHR-5® inhibited virus spread.

When scrutinizing the antiviral potential of *Hamamelis virginiana*, Theisen et al. showed a strong inhibition of influenza A virus A/Puerto Rico/8/34-NS116-GFP with a selectivity index of 85 (EC_{50} 12.1 µg/ml, CC_{50} 1029.1 µg/ml), inhibition of neuraminidase but not hemagglutinin by EGCG (Theisen et al., 2014), which is in accordance with the results presented here, for roseroot tannins. A prodelphinidin-rich extract of *Pelargonium sidoides* impaired influenza viral neuraminidase activity and in contrast to *R. rosea* also hemagglutination (Theisen and Muller, 2012;

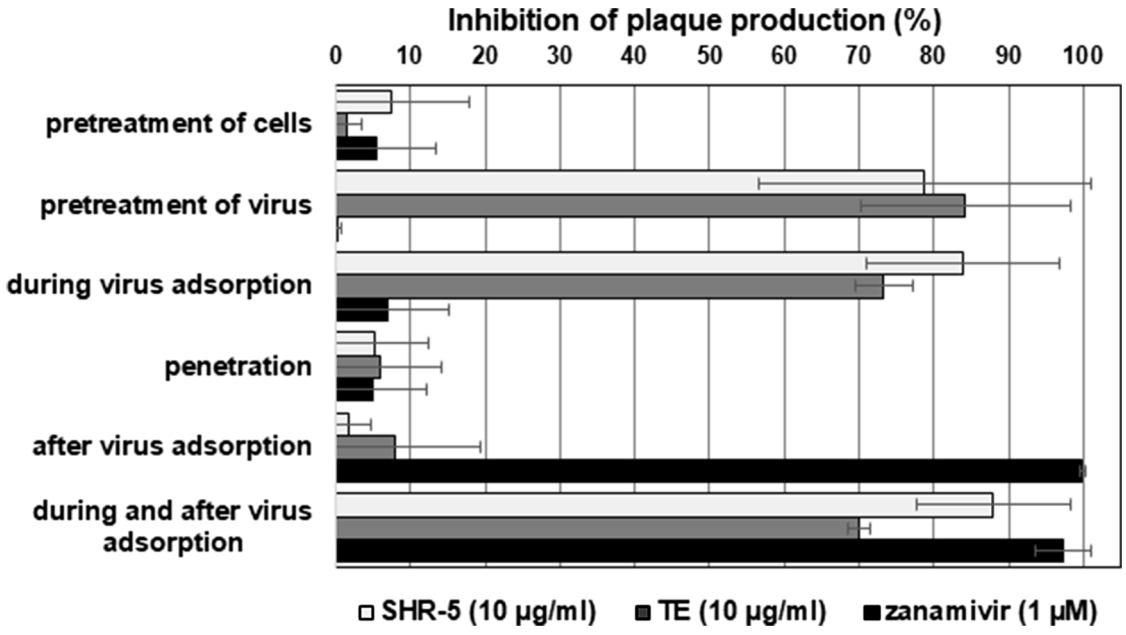

Fig. 4. Effect of SHR-5®, TD, and zanamivir on different steps of the life cycle of influenza virus A/HK/1/68 in MDCK cells. Inhibitors ($n = 4$) were added at different time points before and after infection in plaque reduction assays. Means and standard deviations of the percentage of inhibition of plaque reduction from three independent assays are shown. The results of One-way ANOVA and pairwise multiple comparison procedure (Holm-Sidak method) applied in statistical analysis of data of Fig. 4 are summarized in Table S3.

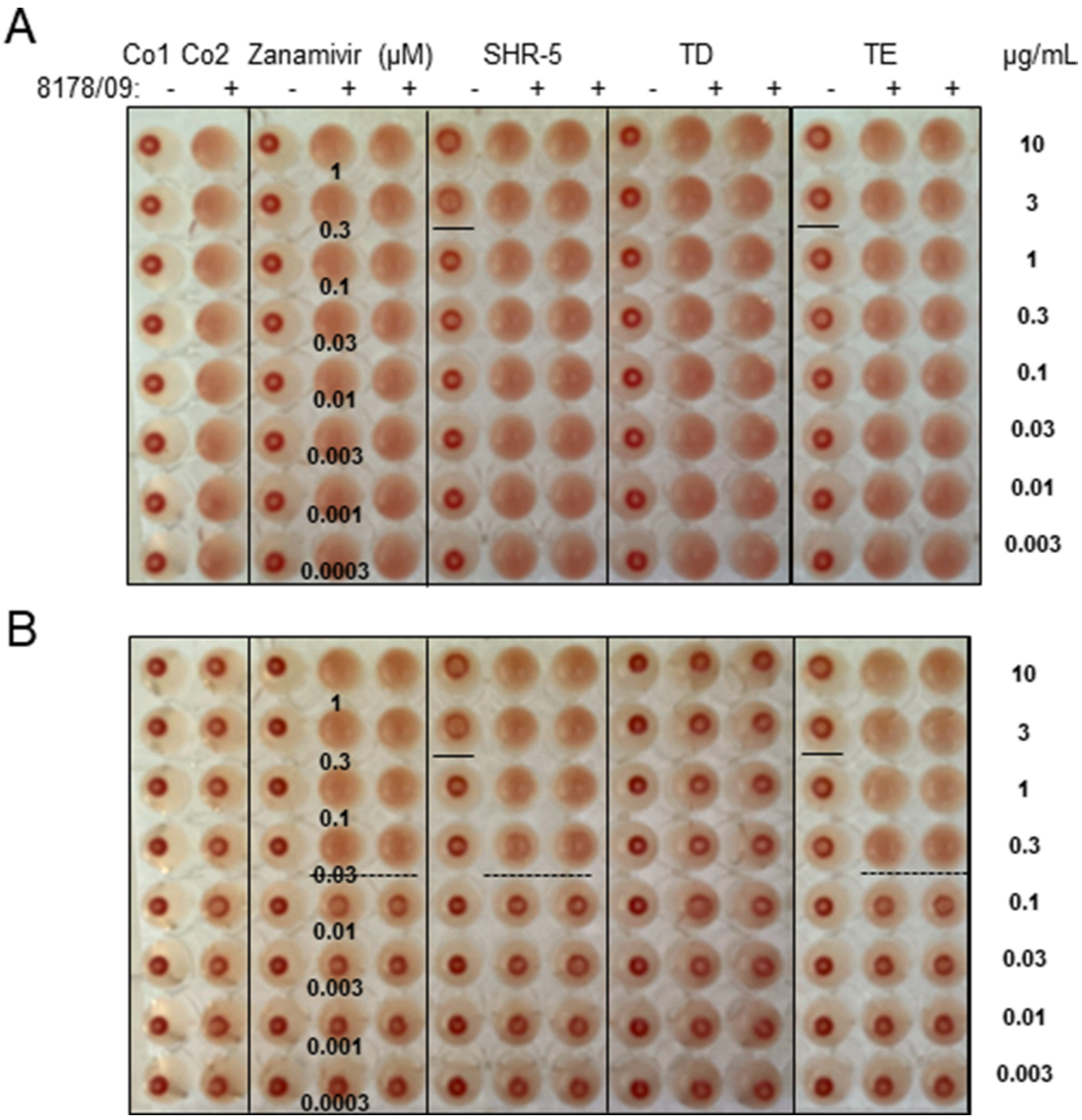

Fig. 5. Influence of SHR-5®, TD, and TE on (A) hemagglutination of human erythrocytes by influenza virus A/Jena/8178/09 (8178/09) and (B) viral neuraminidase activity. (A) Phosphate-buffered saline (Co 1: 8178/09: -) or four hemagglutination units of the virus (Co 2: 8178/09: +) were mixed with phosphate-buffered saline or with serial half-logarithmic dilutions of zanamivir (1 to 0.0003 nM), SHR-5®, TD, and TE (10 to 0.003 µg/ml). A human erythrocyte solution was added before incubating the mixture at 4 °C for 2 h. (B) After protocolling the effect on hemagglutination, we further incubated the test at 37 °C overnight allowing activation of viral neuraminidase activity. Three experiments were performed.

Walther et al., 2020). Whereas Theisen and Muller report an effect in the early step in influenza virus life cycle but no direct virucidal effect (Theisen and Muller, 2012) within another work with *P. sidoides* (Walther et al., 2020) a loss of infectivity was observed.

No resistant virus mutants emerged during treatment with SHR-5®

To study resistance development, we performed four consecutive plaque reduction assays with influenza virus A/BLN/7/2019 and SHR-5® in MDCK cells allowing multiple virus passages. Virus growing in the presence of SHR-5® was picked from plaques. The subsequently determined IC_{50} values of 0.19, 0.25, and 0.26 µg/ml did not show any loss of susceptibility to SHR-5®. Thus, like the *P. sidoides* extract (Theisen and Muller, 2012), SHR-5® showed no propensity to generate resistant viruses. We assume that the multi-functional profile of SHR-5® against influenza virus is responsible for circumventing resistance development.

Conclusion

Extracts of the roots and rhizomes of *R. rosea* have a longstanding tradition as adaptogen, an agent that increases stress resistance in humans by acting against symptoms associated with e.g. anxiety and inadequate sleep (Panossian and Wikman, 2009). In addition to these beneficial adaptogenic properties, we recently described a distinct and specific *in vitro* anti-influenza virus activity for the hydro-ethanolic *R. rosea* extract (SHR-5®). Its flavonoid constituents rhodiosin, tricin, and tricin-5-O-β-D-glucopyranoside moderately contribute to the observed antiviral effect and do not fully explain the antiviral activity of the extract SHR-5® (Langeder et al., 2020b).

Hence, in the present study, a comprehensive *in vitro* anti-influenza virus profile of SHR-5® was assessed for the first time. Taking into consideration possible combination effects of constituents in the extract and the quantitative composition of SHR-5®, we conclude that the pronounced anti-influenza virus activity of SHR-5® can be mostly

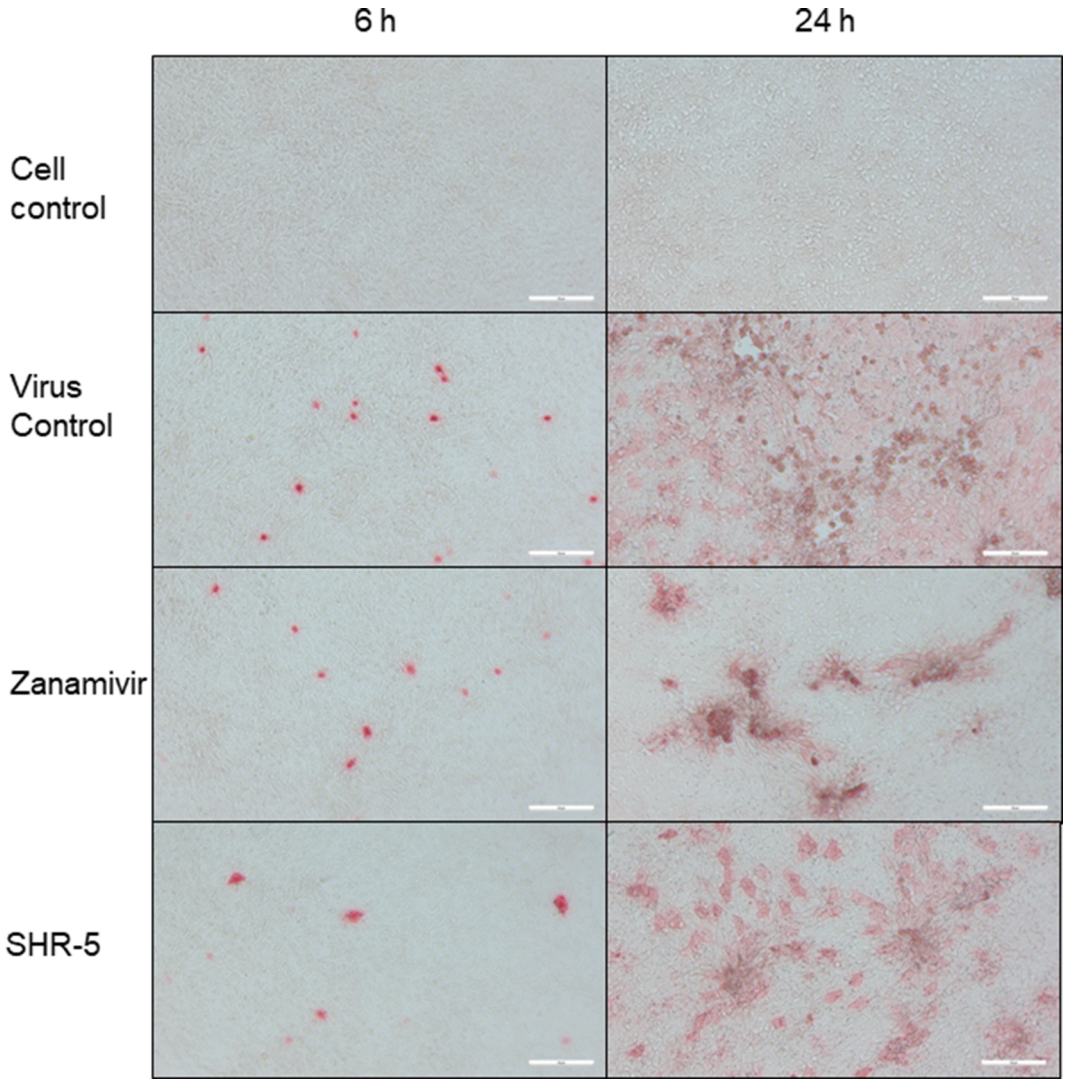

Fig. 6. Inhibition of influenza virus spread in MDCK cells by zanamivir and SHR-5®. MDCK cells (cell control) were infected with influenza virus A/HK/1/68 (multiplicity of infection 0.01 pfu/cell) in the absence of inhibitors for 1 h at 37 °C. After removing non-adsorbed viruses, infected cells were mock-treated with medium (virus control) or treated with zanamivir (1 μM) or SHR-5® (10 μg/ml) in medium for 6 and 24 h. Virus-infected cells were visualized by immunocytochemical detection of viral nucleoprotein (red staining).

explained by the TE fraction mainly composed of prodelphinidin gallate oligomers.

Neither oseltamivir resistance nor M2 ion channel blocker resistance hampered the strong *in vitro* activity of SHR-5® and TE against influenza viruses of the subtypes A and B, which is based on their interaction with the viral envelope also including the inhibition of viral neuraminidase.

In sum, by studying the antiviral mode of action of SHR-5®, a first starting point for its potential application as an anti-influenza herbal remedy has been established. Intriguingly, no development of SHR-5® resistance in influenza viruses was observed *in vitro*, which makes this multicomponent mixture an even more promising therapeutic candidate. After oral administration of SHR-5® in therapeutic doses the maximum concentration of the known adaptogenic compounds salidroside and rosavin has previously been determined in the blood of human subjects with 950 ng/ml and 450 ng/ml, respectively (Panossian et al., 2010a). According to our results and to further ensure reproducible quality with the potential of an additional antiviral activity in all future studies, we advise to include a minimum content of 0.3% of the flavonoids tricin-5-O-β-D-glucopyranoside, tricin, and rhodiosin as analytical marker compounds in the specification and declaration of SHR-5®. Moreover, we suggest a minimum total tannin content of 13%, as TE appears to be a main contributor to anti-influenza virus activity *in vitro*.

Declaration of Competing Interest

The authors declare no conflict of interest.

Author contributions

Kristin Döring: Investigation, Formal analysis, Validation, Writing-Reviewing and Editing Julia Langeder: Investigation, Formal analysis, Validation, Writing- Reviewing and Editing. Susanne Duwe: Investigation, Formal analysis, Validation, Writing- Reviewing and Editing. Ammar Tahir: Investigation, Ulrike Grienke: Conceptualization, Writing- Reviewing and Editing Judith M. Rollinger: Conceptualization, Supervision, Funding acquisition, Writing- Reviewing and Editing Michaela Schmidtke: Investigation, Conceptualization, Supervision, Funding acquisition, Writing - Original Draft. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2021.153895.

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