New Results

Common dandelion (*Taraxacum officinale*) efficiently blocks the interaction between ACE2 cell surface receptor and SARS-CoV-2 spike protein D614, mutants D614G, N501Y, K417N and E484K *in vitro*

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Abstract

On 11th March 2020, coronavirus disease 2019 (COVID-19), caused by the SARS-CoV-2 virus, was declared as a global pandemic by the World Health Organization (WHO). To date, there are rapidly spreading new “variants of concern” of SARS-CoV-2, the United Kingdom (B.1.1.7), the South African (B.1.351) or Brasilian (P.1) variant. All of them contain multiple mutations in the ACE2 receptor recognition site of the spike protein, compared to the original Wuhan sequence, which is of great concern, because of their potential for immune escape. Here we report on the efficacy of common dandelion (*Taraxacum officinale*) to block protein-protein interaction of spike S1 to the human ACE2 cell surface receptor. This could be shown for the original spike D614, but also for its mutant forms (D614G, N501Y, and mix of K417N, E484K, N501Y) in human HEK293-hACE2 kidney and A549-hACE2-TMPRSS2 lung cells. High molecular weight compounds in the water-based extract account for this effect. Infection of the lung cells using SARS-CoV-2 spike

2). It is induced by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Dry cough, fever, fatigue, headache, myalgias, and diarrhea are common symptoms of the disease. In severe cases people may become critically ill with acute respiratory distress syndrome (3). The SARS-CoV-2 virus surface is covered by a large number of glycosylated S proteins, which consist of two subunits, S1 and S2. The S1 subunit recognizes and attaches to the membrane-anchored carboxypeptidase angiotensin-converting enzyme 2 (ACE2) receptor on the host cell surface through its receptor binding domain (RBD). The S2 subunit plays a key role in mediating virus–cell fusion and in concert with the host transmembrane protease serine subtype 2 (TMPRSS2), promotes cellular entry (4). This interaction between the virus and host cell at entry site is crucial for disease onset and progression.

To date there are three rapidly spreading new variants of SARS-CoV-2 which were first reported in the United Kingdom (variant B.1.1.7), South Africa (variant B.1.351) and Brasil (variant P.1), all of which share the mutation N501Y in the spike protein (5). SARS-CoV-2 variants with spike protein D614G mutations now predominate globally. B.1.351 contains, besides D614G, other spike mutations, including three mutations (K417N, E484K und N501Y) in the RBD (6). Preliminary data suggest a possible association between the observed increased fatality rate with the mutation D614G and it is hypothesized that a conformational change in the spike protein results in increased infectivity (7). Free energy perturbation calculations for interactions of the N501Y and

German Commission E, the European Scientific Cooperative for Phytotherapy (ESCOP) monographs (**11**, **12**) as well as in the British Herbal Medicine Association (**13**). The plant contains a wide array of phytochemicals including terpenes (sesquiterpene lactones such as taraxinic acid and triterpenes), phenolic compounds (phenolic acids, flavonoids, and coumarins) and also polysaccharides (**14**). The predominant phenolic compound was found to be chicoric acid (dicaffeoyltartaric acid). The other were mono-and dicaffeoylquinic acids, tartaric acid derivatives, flavone and flavonol glycosides. The roots, in addition to these compound classes, contain high amounts of inulin (**15**). Dosage forms include aqueous decoction and infusion, expressed juice of fresh plant, hydroalcoholic tincture as well as coated tablets from dried extracts applied as monopreparations (**16**) but also integral components of pharmaceutical remedies. Our research was conducted using water-based extracts from plant leaves. We found that leaf extracts efficiently blocked spike protein or its mutant forms to the ACE2 receptor, used in either pre-or post-incubation, and that high molecular weight compounds account for this effect. A plant from the same tribe (*Cichorium intybus*) could exert similar effects but with less potency. Infection of A549-hACE2-TMPRSS2 human lung cells using SARS-CoV-2 pseudotyped lentivirus was efficiently prevented by the extract.

Results

T. officinale inhibits spike S1 – ACE2 binding

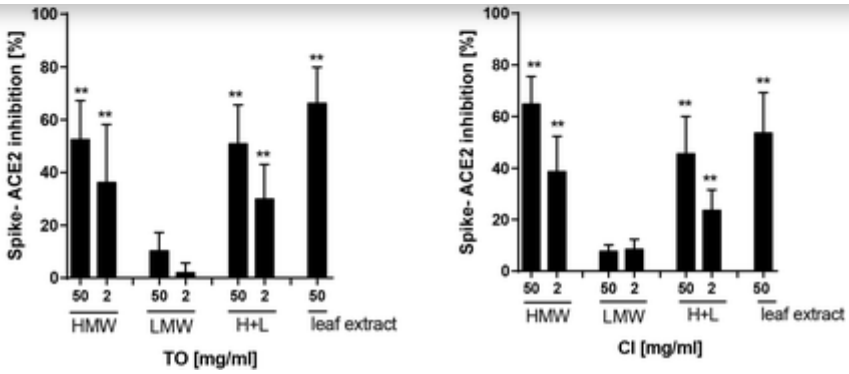

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Figure 1:

Effect of *T. officinale* and chicory on Sars-CoV-2-Spike – ACE 2 inhibition.
A-B) Concentration dependent effect of *T. officinale* (TO) and *C. intybus* (CI) extract. C-D) Effect of fractions from TO and CI leave extract. The extracts were freeze-dried and a molecular weight fractionation subsequently carried out. The cut-off was set to 5 kDa (HMW > 5 kDa, LMW <5kDa). H+L: HMW and LMW fractions; 50 mg of dried leaves per ml water was used as reference. HMW and LMW fraction quantities equivalent to dried leaves were used. The binding inhibition was assessed using ELISA technique. Bars are means + SD. Solvent control: distilled water (a.d.).

Using hACE2 overexpressing HEK293 cells, the potential of *T. officinale* and *C. intybus* extracts to block spike binding to cells was further investigated. As can be seen from **figure 2**, pre-

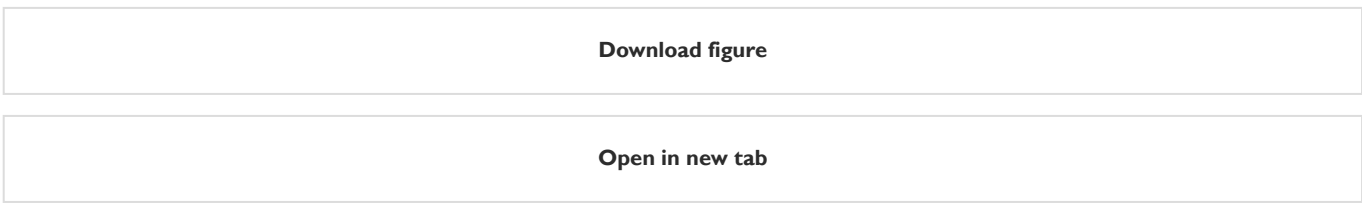

Figure 2:

Binding inhibition of S1 spike protein to human HEK293-hACE2 cells by extract pre-incubation.
Cells were pre-incubated for the indicated times with the extract from 10 mg/ml *T. officinale* (TO), its HMW fraction, equal to 10 mg/ml extract (HMW), and 10 mg/ml *C. intybus* (CI) or solvent control (a.d.) and subsequently treated with HIS-tagged S1 spike protein for 1h without a washing step in between at 4°C. Binding inhibition was assessed using flow cytometry. N=3, bars are means + SD. Upper left: cytogram of gated HEK-hACE2 cells. Middle: overlay of representative fluorescence intensity histograms for ACE2 surface expression. Upper right: overlay of representative fluorescence intensity histograms for spike binding inhibition by the extracts or a.d.; positive control: 20 µg/ml soluble hACE2. Cells were stained with anti-His-tag Alexa Fluor 647 conjugated monoclonal antibody.

Cell treatment with equal amounts of spike D614 and its variants D614G and N501Y confirmed a stronger binding affinity of D614G (about 1.5-fold) and N501Y (about 3 to 4-fold) than D614 spike protein to the ACE2 surface receptor of HEK293 cells (**figure 3A**). Pre-treatment with *T. officinale* quickly (within 30 sec.) blocked spike binding to the ACE2 surface receptor (**figure 3B-C**). After 30 sec., this was 58.2 ± 28.7% for D614, 88.2 ± 4.6% for D614G, and 88 ± 1.3% for N501Y binding inhibition by *T. officinale* extract. Even though for *C. intybus* extract a binding inhibition of spike could be seen, this was about 30-70% less compared to *T. officinale*,

officinale extract blocked binding by 82.97% ± 6.31(extract pre-incubation) and 79.7% ± 9.15 (extract post-incubation).

Spike significantly downregulated ACE2 protein after 6h (4C, black bars), and this was also true for the extract, either alone (4C, white bars) or in combination with spike (black bars). After 24h, this effect was abolished (4D).

Figure 5.

inhibition to ACE2 has not been shown for most of them so far, which might be explained by a lack of complete coverage of ACE2 binding residues by the compounds (**20**). However, for glycyrrhizin, nobiletin, and neohesperidin, ACE2 binding falls partially within the RBD contact region and thus, these have been proposed to additionally block spike binding to ACE2 (**20**). The same accounts for synthetic ACE2 inhibitors, such as N-(2-aminoethyl)-1 aziridine-ethanamine (NAAE) (**21**). In contrast, the lipoglycopeptide antibiotic dalbavancin has now been identified as both, ACE2 binder and SARS-CoV-2 spike-ACE2 inhibitor (**22**); SARS-CoV-2 infection was effectively inhibited in both mouse and rhesus macaque models by this compound. Also, for a hydroalcoholic pomegranate peel extract, blocking of spike-ACE2 interaction was shown at 74%, for its main constituents punicalagin at 64%, and ellagic acid at 36%. Using SARS-CoV-2 spike pseudotyped lentivirus infection of human kidney-2 (HK-2) cells, virus entry was then efficiently blocked by the peel extract (**23**). In the present study, we could show potent ACE2-spike S1 RBD protein inhibition by *T. officinale* extracts using a cell-free assay and confirmed this finding by demonstrating efficient ACE2 cell surface binding inhibition in two human cell lines. We observed stronger binding of the variants D614G and N501Y to the ACE2 surface receptor of human cells, but all tested variants were sensitive to binding inhibition by *T. officinale*, either used before spike protein exposure or thereafter. To date, several studies indicate that the D614G viral lineage is more infectious than the D614 virus (**24**). Also, the presence of characteristic mutations such as N501Y of, e. g. the so-called UK variant B.1.1.7, result in higher infectivity than the

Disruption of the Angiotensin II/Angiotensin-(**1**-**7**) balance by ACE2 enzyme activity inhibition or protein decrease and more circulating Angiotensin II in the system, is e. g. recognised to promote lung injury in the context of COVID-19 disease (**28**, **29**).

The lung would be assumed to be the primary target of interest but, ACE2 mRNA and protein expression have been found in epithelial cells of all oral tissues, especially in the buccal mucosa, lip and tongue (**30**). These data concur with the observation of very high salivary viral load in SARS-CoV-2 infected patients (**31**, **32**). As an essential part of the upper aerodigestive tract, the oral cavity is thus believed to play a key role in the transmission and pathogenicity of SARS-CoV-2. There is high potential that prevention of viral colonization at the oral and pharyngeal mucosa could be critical for averting further infection to other organs and the onset of COVID-19 (**33**). Commercial virucidal mouth-rinses, povidone-iodine at the first place, have thus been suggested to potentially reduce the SARS-CoV-2 virus load in infected persons (**34**-**36**), but significant clinical studies do not exist to date (**36**). Blocking SARS-CoV-2 virus binding to cells of the oral cavity with *T. officinale* extracts might be tolerable for a consumer, if necessary only for limited periods of time (e. g. product application after contact with infected persons or when being infected). More physiologically relevant *in vitro* experiments that were carried out by us showed that only short contact times with *T. officinale* extract were necessary for efficient blocking of SARS-CoV-2 spike binding or for removing already bound spike from the cell surface. Further

The study was carried out using dried leaves from *T. officinale* (vom Achterhof, Uplengen, Germany; batch no. 37259, B370244, and P351756). Plant leaf samples were also collected at three different places in the region of Freiburg i. Br. (Germany), on 12.7.2020, and tested positive in the cell-free spike S1-ACE2 binding assay (data not shown). *C. intybus* was purchased from Naturideen (Germany).

Cell lines and culture conditions

Human embryonic kidney 293 (HEK293) cells, stably expressing hACE2, were generously provided by Prof. Dr. Stefan Pöhlmann (Göttingen, Germany). The cells were maintained in Dulbecco's modified Eagle medium (DMEM), high glucose supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin/streptomycin and 50 µg/ml zeocin (Life Technologies, Darmstadt, Germany). Human A549-hACE2-TMPRSS2 cells, generated from the human lung A549 cell line were purchased from InvivoGen SAS (Toulouse Cedex 4, France) and maintained in DMEM, high glucose supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin/streptomycin, 100 µg/ml normocin, 0.5 µg/ml puromycin and 300 µg/ml hygromycin. To subculture, all cells were first rinsed with phosphate buffered saline (PBS) then incubated with 0.25% trypsin-EDTA until detachment. All cells were cultured at 37 ˚C in a humidified incubator with 5% CO_2/95% air atmosphere.

Plant extraction

ng/ml SARS-CoV-2 Spike -His recombinant protein for 30 min prior to incubation with the plant extract for 30-60 sec at 4˚C or 37˚C. The samples were incubated in PBS buffer containing 5% FCS. Cells were then washed one time with PBS buffer containing 1% FCS at 500 x g, 5 min before staining with His-tag A647 mAb (Bio-Techne GmbH, Wiesbaden-Nordenstadt, Germany) for 30 min at RT. Subsequently, cells were washed twice as described above. The cells were analysed by using a FACSCalibur (BD Biosciences, Heidelberg, Germany), 10.000 events were acquired. The median fluorescence intensity (MFI) of each sample were determined using FlowJo software (Ashland, Oregon, USA).

Human ACE2 enzyme activity and protein quantification

A549-hACE2-TMPRSS2 (2×10^5) cells were seeded in a 24-well plate in high glucose DMEM medium, containing 10% heat inactivated FCS, at 37˚C, 5 % CO_2. Cells were then treated with *T. officinale* extract with/without 500 ng/ml SARS-CoV-2 S1 Spike RBD protein for 1-24h. Afterwards, cells were washed with PBS and lysed. 25µg protein were used for quantification of ACE2 protein (ACE2 ELISA kit), 5 µg for ACE2 enzyme activity (ACE2 activity assay kit, Abcam, Cambridge, UK) according to the manufacturer's instructions.

Infection of A549-hACE2-TMPRSS2 cells using SARS-CoV-2 pseudotyped lentivirus

After 24h SARS-CoV-2 spike pseudotyped lentivirus transduction and 60h post-infection of A549-hACE2-TMPRSS2 cells, supernatants were collected and stored at −80 ˚C until analysis for cytokine secretion using the human MACSplex cytokine 12-kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to manufacturer's protocol.

Molecular weight fractionation from plant extracts

Extracts from dried plant leaves were prepared by adding bidistilled water (5 ml) to plant material (500 mg each). The samples were incubated in the dark at room temperature (RT) for 60 min, followed by centrifugation at 16.000 g for 3 min. The supernatants were collected and membrane filtrated (0.45 µm), resulting in the extracts. Aliquots were freeze dried for 48h to determine their yield by weight. The extracts were then further separated in a high molecular weight (HMW) and low molecular weight (LMW) fraction, using a centrifugation tube with an insert containing a molecular weight cut-off filter (5 kDa, Sartorius Stedim Biotech, Goettingen, Germany). Each HMW fraction was purified by flushing with 20 ml of water, yielding the HMW fractions, as well as LMW. The fractions were freeze dried, their yield determined by weight and stored at −20˚C until use.

Determination of cell viability using trypan blue staining

Cell viability was assessed using the trypan blue dye exclusion test as described before (Odongo et al., 2017). Briefly, A549-hACE2-TMPRSS2 cells were cultured for 24h, and then exposed to extracts or the solvent control (a. d.) for 84 h.

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