1 **Title:**

2 *In vitro* screening of anti-viral and virucidal effects against SARS-CoV-2 by *Hypericum*

3 *perforatum* and *Echinacea*.

4 **Author:**

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Abstract

Special Infectious Agent Unit in King Fahd Medical Research Center at King Abdulaziz University, Jeddah, Saudi Arabia, has pursued the anti-viral project field to optimize the group of medicinal plants for human-infectious diseases. We have begun virtually in this field since COVID-19 pandemic, besides our divergence in the infectious agents'. In this study and based on the previous review, *Hypericum perforatum* (St. John's Wort) and *Echinacea* (gaia HERBS®) were tested *in vitro* using Vero E6 cells for their anti-viral effects against the newly identified Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) through its infectious cycle from 0 to 48 hours post infection. The hypericin (0.9 mg) of *H. perforatum* and the different parts (roots, seeds, aerial) of two types of *Echinacea* species (*Echinacea purpurea* and *Echinacea angustifolia*) were examined their efficacy in certain concentration and under light-dependent anti-viral activities to measure the inhibition of the SARS-CoV-2 mRNA expression of RNA-dependent RNA polymerase (RdRP) gene and the viral load with quantitative real-time polymerase chain reaction (qRT-PCR), and to assess the neutralization of the SARS-CoV-2 spike receptor binding on cell culture assay. Interestingly, the mixture (*H.E.*) of 100 mg/mL of *H. perforatum* and *Echinacea* was tested too on SARS-CoV-2 and showed crucial anti-viral activity competing *H. perforatum* then *Echinacea* effects as anti-viral treatment. Therefore, the results of gaia HERBS® products, *H. perforatum* and *Echinacea* species, applied in this study showed significant anti-viral and virucidal effects in the following order of potency: *H. perforatum*, *H.E.*, and *Echinacea* on SARS-CoV-2 infectious cycle; and will definitely required a set up of clinical trial with specific therapeutic protocol based on the outcome of this study.

Author Summary

After an outbreak of Rift Valley Fever in the Southern region of Saudi Arabia, particularly in May 2003, Special Infectious Agents Unit (SIAU) was established and founded by Prof. Esam Ibraheem Azhar. This unit contains a full range of facilities including Biosafety Level 3, allows him and his research groups to ambulate and culture risk group 3 viruses in Saudi Arabia & Gulf States for the first time. Since that time, SIAU and our international collaboration have been extended to implement a standard protocols in the infectious agents diagnostics procedure through different mode of collaboration including exchange of expertise, joint research program and more recently a technology transfer agreements with number of international institute sharing same interests. Furthermore, we have been engaged in number of researches related to Hajj & Umrah plus number of national services with the Ministry of Health (MOH) through which, we utilize our Mobile biosafety level 3 Lab to enhance the diagnostics of MERS CoV in the Holly sites during Hajj since 2014 .

In our SIAU and with a powerful team, we have excellent researches made valuable contributions through *in vivo* and *in vitro* animal and human studies, and several human viral pathogens which are a threat to global health security due to millions of pilgrims visiting Saudi Arabia every year from 182 countries: with particular areas of interests in: Alkhurma Viral Hemorrhagic Fever, Dengue Hemorrhagic Fever Viruses, Rift Valley Fever Virus, MERS-CoV and more recently the new global infectious diseases threat, Sever Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2).

Introduction

Coronaviruses (CoVs) are a large group of RNA viruses with single-stranded RNA genomes that cause 30% of upper and lower respiratory tract infections in humans. Four main human coronaviruses (HCoVs) are known including severe acute respiratory syndrome SARS-CoV [1], HCoV-NL63 [2], HCoV-HKU1 [3], and MERS-CoV in 2012 [4]. SARS-CoV-2 originated from Wuhan, Hubei Province of China and spread to the rest of the world causing the global pandemic of COVID-19 with symptoms ranging from cough, fever, and dyspnea to neurological and vascular symptoms [5]. With advancement of the disease, intensive production of pro-inflammatory cytokines findings like: TNF- α , IL-1 β , IL-6, IFN- γ , CXCL10, MCP-1, resulting in vasculitis, hyper-coagulability, and multi-organ damage which lead ultimately to death [6]. Main protease (Mpro), known as 3-chymotrypsin-like cysteine protease (3CLpro), has been considered as an important functional target in the viral life cycle, and therefore as a candidate target for anti-viral drugs against SARS-CoV-2 [7, 8] due to its role in the release of functional polypeptides that is encoded by all HCoVs [9, 10]. Updated reports about the disease management from COVID-19 have targeted its structure, pathology, and mechanism in order to have the best solution against the infection by both redesigning and using classical anti-inflammatory agents and by targeting certain cytokine of molecular drug, as per WHO recommendation [11]. For example, numbers of feasible treatment against SARS-CoV-2 were proposed such as: neuraminidase inhibitors, Remdesivir, Peptide (EK1), abidol, RNA synthesis inhibitors (TDF and 3TC), anti-inflammatory drugs (hormones and other molecules), and Chinese traditional medicine [12]. Furthermore, there were several studies about the synergy between antimalarial drugs, like Chloroquine-Hydroxychloroquine, Remdesivir-Favipiravir for the treatment of COVID-19 [13-15].

H. perforatum (family Hypericaceae), or St. John's Wort (SJW) has been very well known for a long time as an effective medicinal plant for a range of communicable and non-communicable diseases such as depression, bacterial and viral infections, skin wound, and inflammation [16, 17]. Herbal medicine studies demonstrate the pharmacological efficacy against microbial infections [16, 18, 19]. As an anti-viral agent, *H. perforatum* activities were assessed *in vitro* and *in vivo* on infectious bronchitis virus (IBV), Hepatitis C, HIV and Coronaviruses other than SARS-CoV-2 [20, 21]. Several Studies showed its activity against *Escherichia coli*, *Shigella dysenteriae*, *Salmonell typhi*, *Bacillus cereus*, and several oral bacteria [22]. Because of *H. perforatum's* metabolites from each extracted part (roots, seeds and aerial), they are chemically defined to naphthodianthrone (*hypericin*), phloroglucinols (*hyperforin*), flavonoid glycosides (*hyperoside*), biflavones, and anthocyanidins [19]. Therefore, the hyperforin and hypericin of *H. perforatum* are shown to be effective as antibacterial compounds against various Gram-positive bacteria but ineffective against Gram-negative bacteria [23-26]. Furthermore, ethyl acetate extraction section of Hypericum showed a significant reduction on the titer of IBV [16].

Another medicinal plant, which was applied for many traditional and common remedies like curing cold and flu symptoms and boosting immune system, is *Echinacea*. *Echinacea* is known with nine species of several plants in the genus of *Echinacea*, however, only three of them were used as herbal complements: *E. angustifolia*, *E. purpurea*, and *E. Pallida*. *Echinacea* contains chemical compounds liable for medicinal properties as: caffeic acid derivatives, polysaccharides, flavonoids, ketones, and lipophilic alkalamides [27-34]. According to *in vitro* and *in vivo* studies, *Echinacea* showed effects on the cytokine production [35, 36], increasing the expression of CD69 [37], an impact on natural killer cells [38], as well as reducing illness severity, *in vivo*, as anti-inflammatory therapeutic agents on human monocytic THP-1 cells [39]. The latter is due to its efficacy on upper respiratory tract

infections especially resulted from *E. purpurea* root derived a polysaccharide-containing water, and thereby activates phosphatidylinositol-3-kinase (PI3K) of following inhibition of TLR1/2 mediated stimulation of TNF α production [39]. Also, alkylamides and ketones of *Echinacea* extracts reported the anti-inflammatory effects [30, 40-42]. Another study in 2009 against H5N1 HPAIV strain showed that the extract of *E. purpurea* interferes with the viral entry into cells by blocking the receptor binding activity of the virus [43].

The application of medicinal plant extracts is still a controversial issue especially the plant extracts found in different manufacturing methods with varying use of plants parts and species. In this study, we evaluated the efficacy of *H. perforatum* and *Echinacea* as anti-viral agents against SARS-CoV-2 *in vitro*. The *H. perforatum* and *Echinacea* species applied in this study included aerial parts with high concentration of hypericin (main component 0.9 mg) and three different parts of type *E. purpurea* (root, seed, aerial parts) and one part of type *E. angustifolia* (root).

Results

Evaluation of Cytotoxicity of *Hypericum perforatum* and *Echinacea*

The cytotoxicity of *H. perforatum*, *Echinacea* and the *H.E.* mixture of both was evaluated on Vero E6 cell 48h post-treatment using MTT assay. The results showed that most of the plant extracts displayed minimal cytotoxicity at concentrations less than 12.5 µg/mL compared to the cell control (Figure 1). Also, the maximum non-toxic concentrations of *H. perforatum*, *Echinacea* and *H.E.* mixture were found to be: 1.56, 6.25, and 6.25 µg/mL; respectively.

Figure 1. Cell viability of Vero E6 was determined by MTT assay. The survival rate of cells treated with different concentrations of *H. perforatum*, *Echinacea* and *H.E.* ranging from 100 to 0.78 µg/mL. The percentage cell viability was calculated relatively to cell control wells. More than 50% cell survival rate was considered to be the maximum non-toxic concentration of *H*, *E* and *H.E.* Statistical analysis showed that data were significant with $p < 0.005$ (one-way ANOVA) of three independent experiments.

Serial dilutions ranging from 100-0.78 $\mu\text{g/mL}$ were chosen for *in vitro* micro-inhibition assays, so, *H. perforatum* and *H.E.* showed IC_{50} values of 7.724 and 8.202 $\mu\text{g/mL}$, respectively, while the effect of *Echinacea* was not concentration dependent as shown in Table 3 and Figure 2.

Figure 2. Dose-response curve analysis by Graph pad software for *H. perforatum*, *Echinacea* and *H.E.* activities. The black curve represents inhibition of SARS-CoV-2 infection (%) and the red dots represent log concentration ($\mu\text{g/mL}$).

Table 3. Cellular toxicity and maximum non-toxic concentrations of *H. perforatum*, *Echinacea* and *H.E.* mixture against SARS-CoV-2 infection.

Medicinal plant	IC_{50} ($\mu\text{g/mL}$)	Max. non-toxic conc. ($\mu\text{g/mL}$)
H	7.724	1.56
E	Nd	6.25
HE	8.202	6.25

Anti-viral efficacy of *Hypericum perforatum* and *Echinacea*

The effect of the medicinal plants was evaluated by the anti-viral assays as following:

1. Direct treatment of virus-infected cells assay: In this assay the virus was incubated with cells for two hours at 37°C prior adding the medicinal plant mixture to the cells. Results from this assay showed that *H. perforatum* had the highest efficacy with IC_{50} of 1.56 $\mu\text{g/mL}$ while *H.E.* mixture with IC_{50} of 6.25 $\mu\text{g/mL}$ and the least effective was *Echinacea* of 6.25 $\mu\text{g/mL}$. Following the viral load with time (12, 18, 24, 36, 48hrs) after addition of the mixture showed a significant reduction of the viral load for *H. perforatum* up to 36hrs

of addition ($p=0.0047$) followed by *H.E.* mixture up to 36hrs of addition ($p=0.0048$) and *Echinacea* up to 24hrs of addition ($p=0.0060$).

2. Pre-treatment of cells prior viral infection assay: In this assay the cells were incubated with medicinal plants for two hours at 37°C prior viral infection of the cells. The outcome of this assay showed that *H. perforatum* had an effective concentration of 1.56 µg/mL, while *H.E.* mixture had an effective concentration of 6.25 µg/mL and the least effective was *Echinacea* with 6.25 µg/mL concentration. Figure 4 showed the reduction in the viral load with *H. perforatum*, *H.E.* mixture and *Echinacea* over time (12hrs - 48hrs).
3. Virucidal activity assay: in this assay the virus was incubated with the medicinal plants for two hours prior addition to the cells. Results from this assay showed that *H. perforatum* had the highest effect of 1.56 µg/mL followed by the mixture *H.E.* of 6.25 µg/mL and the least effective was *Echinacea* of 6.25 µg/mL. The time of addition showed a significant reduction in the viral load for *H. perforatum* that lasted longer than 48hrs, and this result shown as well with *H.E.* mixture; and finally *Echinacea* displayed the best virucidal effect up to 36hrs.

The anti-viral effect of *H. perforatum*, *Echinacea* and *H.E.* (Figure 3 & 4) was obvious under maximum non-toxic concentration 1.56, 6.25, and 6.25 µg/mL, respectively, and the inhibition of *H. perforatum* on SARS-CoV-2 was the greatest from *H.E.* and *Echinacea* in the three anti-viral assays. Also, *Echinacea* was a weaker inhibitor than the *H. perforatum* and *H.E.* but slightly strong as a virucidal up to 24hrs.

Figure 3. The inhibitory effect of *H. perforatum*, *Echinacea*, and *H.E.* on mRNA expression levels of SARS-CoV-2 RdRP gene in Vero E6 cells was evaluated by qRT-PCR. The impact of plant materials was detected by the three experimental designs: Direct Treatment of Virus-infected Cells (A), Pre-treatment of Cells Prior to Virus Infection (B), and Virucidal (C) assays; treated and infected cells were included as negative and positive controls, respectively. So, 100 TCID₅₀ of the virus was added with the certain concentrations of the plant materials when the cells at 80% confluence were complete. The viral inhibition percentage was calculated relatively to virus control wells, and representatives of two independent experiments performed in triplicate are shown. Statistical analysis showed that data were significant with $p < 0.05$ and $p < 0.005$ (one- & two-ways ANOVA).

Figure 4. The effect of *H. perforatum*, *Echinacea*, and *H.E.* on viral load of SARS-CoV-2 infection in Vero E6 cells was evaluated by qRT-PCR. The impact of plant materials was detected by the three experimental designs: Direct Treatment of Virus-infected Cells (A), Pre-treatment of Cells Prior to Virus Infection (B), and Virucidal (C) assays; treated and infected cells were included as negative and positive controls (PC), respectively. So, 100 TCID₅₀ of the virus was added with the certain concentrations of the plant materials when the cells at 80% confluence were complete. The effect of the plant materials was observed clearly with reduction in viral load at 1.56, 6.25, and 6.25 µg/mL, respectively. The viral load was calculated relatively to virus control wells, and representatives of two independent experiments performed in triplicate are shown. Statistical analysis showed that data were significant with $p < 0.005$ (one- & two-ways ANOVA).

Discussion 252

COVID -19 pandemic is causing a global challenge to the world economic and 253
healthcare systems. The pandemic is responsible for the death of over 1.8 million confirmed 254
cases and 85.5 million infections worldwide (WHO). Although some vaccines are developed 255
and are now released under emergency use because of the pandemic, the efficacy of the 256
vaccines is still debatable specially with the emergence of new variants in the genomic 257
structure [44-47]. Several non-specific treatment options were evaluated and entered clinical 258
trial including the repurposing of known treatments against other diseases [48]. Therefore, 259
improvement and investigation of an effective anti-viral therapy is a for treating SARS-CoV- 260
2 infection. 261

In our study, the anti-SARS-CoV-2 effect of the medicinal plants *H. perforatum*, 262
Echinacea and their combination was evaluated. The medicinal plants were purchased from a 263
commercial source to ensure consistency of the composition and were tested either 264
individually or combined together as a single treatment. Their mode of action was evaluated 265
using three approaches namely direct treatment of virus-infected cells, pre-treatment of cells 266
prior viral infection and virucidal approaches. The results were evaluated using qRT-PCR 267
assay relative to the virus control (virus added to the cells without the medicinal plants) and 268
were observed under the microscope for CPE effect on the cells and by colorimetric staining 269
using crystal violet Figure 5. 270

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A	24H	36H	48H	B	24H	36H	48H	C	24H	36H	48H
H				H				H
E				E				E
HE				HE				HE

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Figure 5. The impact of *H. perforatum*, *Echinacea* and *H.E.* on SARS-CoV-2 infected-Vero E6 cells by anti-viral activity essays. The cell viability and CPE through SARS-CoV-2 infectious cycle, from 24 to 48 hours post infection, were visualized by photographed images with a light microscope (magnification, x100) (orange squares) and by the crystal violet assay (blue circles). The images were captured when the CPE started on the positive control at 24hrs.

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The cytotoxic effect of the tested medicinal plants was evaluated using MTT assay with results presented as percent of cytotoxicity relative to cell control (cells with no added tested medicinal plants). The IC_{50} of *H*, *E* and *H.E.* mixture was as follows 7.724, not detected and 8.202; respectively.

Our results demonstrated that the *H. perforatum*, containing 0.9 mg of hypericins, can significantly reduce SARS-CoV-2 viral load compared to the positive control (Table 4, Figure 3 & 4) through the viral infectious cycle from 0hr to 48hrs, and clearly this reduction in the anti-viral (treatment of virus-infected cells and pre-treatment of cells prior to virus infection) and virucidal assays .

Table 4. Inhibitory effects of *H. perforatum*, *Echinacea* and *H.E.* on SARS-CoV-2 mRNA expression level of RdRP gene through infection in Vero E6 cells.

Plant materials	Direct Treatment of Virus-infected Cells (Anti-viral Activity)				
	Viral Inhibition (%)				
	12h	16h	24h	36h	48h
H	0.64	1.45	5.54	17.75	13.76
E	-3.02	-2.92	-5.20	-1.57	0.05
HE	-2.31	2.52	2.09	6.02	6.51
PC (SARS-CoV-2)	13.72	10.46	-2.85	-21.13	-25.73

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Plant materials	Pre-treatment of Cells prior to Virus Infection (Anti-viral Activity)				
	Viral Inhibition (%)				
	12h	16h	24h	36h	48h
H	5.32	5.96	17.67	29.64	25.22
E	0.36	0.18	4.52	1.53	3.29
HE	4.84	2.86	10.84	16.11	22.58
PC (SARS-COV-2)	13.72	10.46	-2.85	-21.13	-25.73

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Plant materials	Pre-treatment of Virus Prior to Infection (Virucidal Activity)				
	Viral Inhibition (%)				
	12h	16h	24h	36h	48h
H	4.41	9.93	18.62	35.33	35.77
E	3.56	4.34	9.04	3.30	4.50
HE	5.86	8.75	19.06	31.36	27.07
PC (SARS-COV-2)	13.72	10.46	-2.85	-21.13	-25.73

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Noticeably the results showed that the highest anti-viral effect was through the virucidal mechanism (Figures. 4 and 6) compared to the other two mechanisms with the highest inhibition observed at 36hrs after infection as shown in Figure 3. While, *H. perforatum* has the strongest inhibitory effect (35.77%) and reducing the viral load, up to 48hrs, compared to *Echinacea* and *H.E.* mixture that had 3.30 and 31.36%, up to 36hrs, respectively.

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The anti-viral effect of the *H. perforatum* (with concentration of 1.56 µg/mL) was higher than *Echinacea* and *H.E.* mixture (with concentration of 6.25 µg/mL for both), and the effect was noted at lower IC₅₀ and a higher reduction in viral load of SARS CoV-2. *H. perforatum* showed the highest inhibitory effect of in all three anti-viral assays Figure. 6, while *Echinacea* showed the lowest inhibitory effect. The *H.E.* mixture showed lower inhibition than *H. perforatum* alone that would be expected as the active compounds were diluted by the addition of *Echinacea*.

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Hypericins were reported to be the active anti-viral compounds in *H. perforatum* extract against IBV *in vitro* and *in vivo* [14]. The *H. perforatum* ethylacetate (HPE) extract includes: hyperoside, quercitrin, quercetin, pseudohypericin, and hypericin. The anti-viral effect of HPE was reported previously against bovine diarrhea virus (BVDV) *in vitro* [48] and hepatitis C virus (HCV) *in vivo* [49] with hypericin of *H. perforatum* extract defined as the active ingredient. Other studies in 2001, 2009, and 2012 showed that *H. perforatum* extract had an anti-viral effect against influenza A virus and HIV [50-52], suggesting that HPE could be developed and used as an anti-viral drugs. In addition, the anti-viral effect of *H. perforatum* extract and HPE had a remarkable decrease in the concentration of IL-6 and TNF-α through the NF-κB in lung tissue of mice infected with an influenza A virus [52] and in the trachea and kidney for chickens infected with IBV, mainly from hypericin content [14].

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Echinacea, as indicated by the supplier insert is composed of two types (*E. purpurea* 333 (root, seed, aerial parts) and *E. angustifolia* (root)). The beneficial effects of *Echinacea* were 334 reported previously in both *in vitro* and *in vivo* studies as preventive and treatment for 335 respiratory tract viruses due to its active components such as: polysaccharides, glycoproteins, 336 caffeic acids, and alkamides [53-56]. Also, *Echinacea* was reported to achieve the inhibition 337 against influenza A and B, respiratory syncytial virus (RSV), parainfluenza virus, and herpes 338 simplex virus [41, 57]. *E. purpurea* was also demonstrated to interfere with H5N1 and 339 HPAIV cellular entry into cells by blocking the receptor binding activity of the virus, and to 340 emphasize the direct contact with it and the virus in order to gain the maximum inhibition of 341 replication [41]. Because of *Echinacea's* reduction on virus cytokine liberation, it can shorten 342 and relieve the severity of symptoms as a result of pro-inflammatory cytokines release [58, 343 59]. An *in vivo* study is needed to evaluate the effect of *Echinacea* on cytokine regulating 344 during COVID-19 infection together with the anti-viral effect found in this study of *H.* 345 *perforatum*. Lately, Signer and his group showed *in vitro* that Echinaforce®, type of *E.* 346 *purpurea* and from a commercial product, is virucidal against human coronavirus (HCoV) 347 229E, MERS- and SARS-CoVs, as well as SARS-CoV-2 [60]. In hence, gaia HERBS® 348 *Echinacea* has consistent result as an effectively prophylactic treatment in virucidal by 349 blocking the receptor binding activity of the virus; and anti-viral activities (pre-treatment of 350 cells prior to virus infection) by inhibition of the viral load, *in vitro* against SARS-CoV-2 351 infection. 352

An individual finding of our study, the application of both *H. perforatum* and 353 *Echinacea* (100 mg/mL, of each) exerted the high inhibition in the two anti-viral and 354 virucidal activities against SARS CoV-2 infection cycle up to 48hrs (Table 4, Figure 3). So, 355 this result provides, for the first time, clear evidence that the mixing materials of *H.* 356 *perforatum*, containing pseudohypericin, hypericin, hyperforin, adhyperforin, quercetin, 357

quercitrin, and other components, possess anti-SARS CoV-2 activities with the components 358
of *Echinacea* (Table 2). In addition, the mixture effects correlated with reducing the viral 359
load up to 48hrs post infection (Figure 6). 360

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Figure 6. The effect of *H. perforatum*, *Echinacea*, and *H.E.* on viral load of SARS-CoV-2 infection in Vero E6 cells was evaluated by qRT-PCR. The impact of plant materials was detected by the three experimental designs: Direct Treatment of Virus-infected Cells (A), Pre-treatment of Cells Prior to Virus Infection (B), Virucidal (C) assays, and positive control (PC). The effect of the plant materials was observed clearly with reduction in viral load at 1.56 µg/mL for *H. perforatum*, then for *H.E.* at 6.25 µg/mL, and finally for *Echinacea* at 6.25 µg/mL.

For clinical trial, the mixture may empower the inhibition of the virus by up-regulating the mRNA expression process, lower the viral load, and neutralizing the virus envelop receptor as anti-viral or/and virucidal activities, respectively; and definitely it is related to the pro-inflammatory cytokines such as: IL-6, TNF-α, INF-β as anti-inflammatory therapy. It can be useful to precede and develop a new technique for treatment SARS-CoV-2 patients in mild or sever cases, protecting people who may contact infected patients, or/and having positive COVID-19 test. In addition, regarding the using of mixture materials in clinical trial, it was clear that preparation 100 mg/mL of each medicinal plant was safe *in vitro* rather than 200 mg/mL in testing the efficacy against SARS CoV-2 infection, which was recommended using *H. perforatum* and *Echinacea* for patients in different-time administration. Alternatively, *H. perforatum* is preferable single used as anti-viral treatment for the mild to sever case-patients because it demonstrated a high efficacy against SARS-CoV-2 infection. On the other hand, either *Echinacea* or *H.E.* is suggested to be admitted as prophylactic treatment with considering the dose, side effects, drug interactions, and toxicity.

Materials and Methods

Cell Cultures

Vero E6 cells (ATCC® CRL-1586™) were maintained in 75 cm² cell culture flask containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (SIGMA) at 37 °C in an incubator containing 5% CO₂. For preparation 96-well plates with Vero E6 cells, the plates were seeded with 3.10⁴ cells/well and incubated for 24hrs at 37°C with 5% CO₂ until a confluent monolayer was attained [61].

Viral isolation and stock

The procedure of viral isolation of our human SARS-Cov 2 clinical patient isolate (SARS-CoV-2 /human/SAU/85791C/2020, gene bank accession number: MT630432) was described in [62]. The viral production was done in 75 cm² cell culture flask containing Vero E6 cells in Minimum Essential Media (Gibco, ThermoFischer) (MEM) with 4% of fetal bovine serum and 1% glutamine. Cytopathic effect was monitored daily under an inverted microscope. In approximately 72hrs, nearly complete cell lysis was observed of the TCID₅₀ of the strain at 3.16.10⁶ infectious particles per mL. The viral supernatant was used for inoculation on 96-well plate.

Commercial Products of *Hypericum perforatum* and *Echinacea* Extracts

H. perforatum and *Echinacea* products were purchased from gaia HERBS® as gelatin capsules and as liquid; respectively. Table 1 shows the full the information provided on the packaging of products along with information leaflets. The products were sold as dietary and herbal supplements carried the Certified B Corporation logo which transmits a level of quality (Table 1, 2).

Table 1. The products materials used from gaia HERBES®.

Proprietary Extract Blend	Amount per serving	ĖHerb Equivalency
St John's Wort (<i>Hypericum perforatum</i>) aerial parts, St John's Wort (<i>Hypericum perforatum</i>) aerial parts extract.	500 mg	Hypericins (0.9 mg)
Organic <i>Echinacea purpurea</i> root, Organic <i>Echinacea angustifolia</i> root, Organic <i>Echinacea purpurea</i> seed, Organic <i>Echinacea purpurea</i> Aerial parts.	2 mL	444 mg/mL

Table 2. Components found in *Hypericum perforatum* aerial part and *Echinacea* species.

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<i>Hypericum perforatum</i>	<i>Echinacea</i> species
Aerial parts: naphthodianthrones (hypericin and pseudohypericin), acylphloroglucinols (hyperforin and adhyperforin), flavonol glycosides (quercetin, quercitrin, isoquercitrin, rutin, hyperoside, kampferol, leteolin, myricetin), biflavones (^{13,118} -biapigenin, ^{139,118} - biapigenin (amentoflavone)), Proanthocyanidins (procyanidin B2) and phenyl propanes (chlorogenic acid and caffeic acid) [65, 66]	<i>Echinacea angustifolia</i> root [63] Essential oil Polyacetylenes Alkamides Alkaloids Caffeic acid derivatives Polysaccharides and glycoproteins <i>Echinacea purpurea</i> root [63] Essential oil Alkamides Alkaloids Caffeic acid derivatives Polysaccharides and glycoproteins <i>Echinacea purpurea</i> seed [64] Essential oil Fatty acids [64] <i>Echinacea purpurea</i> aerial parts [63] Essential oil Alkamides Flavonoids and anthocyanins Caffeic acid derivatives and phenolic acids Polysaccharides

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Sample Preparation

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Gelatin capsules were separated and the contents emptied into a weighing boat to 415
ensure the coating was not included. Then, the plant materials, capsule sample and liquid, in 416
concentration of 100 mg/mL [67] were dissolved in Dimethyl sulfoxide (DMSO, ≥99.5%, 417
plant cell culture tested, SIGMA) and filtered into 0.22 µm [68]. All the extracts were filtered 418
to remove any plant fibers. The mixture (*H.E.*) of *H. perforatum* and *Echinacea* was prepared 419
by mixing each plant material at 100 mg/mL. Finally, the stocks were stored at -20 °C. 420

Cytotoxicity Assay

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The cytotoxicity assays were performed by 3-(4,5-dimethylthiazol-2-yl)-2,5- 422
diphenyltetrazolium bromide (MTT, Roche, ref:11465007001) protocols according to the 423

previous study [47] with few modifications. Vero E6 cell monolayers with 4.10^3 cells/mL plated onto 96-well culture plates were washed 3 times with phosphate buffer saline (PBS) 1x, pH 7.4. 100 μ l of prepared *H. perforatum*, *Echinacea* and *H.E.* diluted in serum-free DMEM medium (two-fold dilutions, ranging from 0.78-100 μ g/mL) and another 100 μ l of growth medium added onto the wells (3 wells per each dilution). Control cells were incubated with the same corresponding concentrations of DMSO solutions as negative controls. The cells were cultured at 37°C for 48h in a CO₂ atmosphere, and the supernatant was taken out and washed 3 times with PBS, then 20 μ L of MTT solution was added to each well and incubated for 4hrs at 37°C. Solubilization solution was added and incubated for overnight at 37°C. Then, the evaluation of the plate was done using a reader (Synergy 2 microplate, BIOTIK) with a reference wavelength of 570 nm, and the OD570 was recorded. Cell survival rates were calculated from mean values according to the following equation: $(OD570 \text{ drug}) / (OD570 \text{ control}) \times 100\%$.

***In vitro* Micro-inhibition Assay**

According to a method described previously [69] and with some modifications, the plant materials activity against SARS-CoV-2 was evaluated from the curve that was generated by plotting percentages of virus inhibition against concentrations of plant materials. Briefly, 96-well plates were prepared as above mentioned with Vero E6 cells. Then, the cells were washed twice with PBS, and two-fold serial dilutions of plant materials (*H. perforatum*, *Echinacea* and *H.E.*) (0.78-100 μ g/mL) in medium were challenged with 100 TCID₅₀ of SARS-CoV-2 and incubated for three days at 37°C/5% CO₂, the results were quantified as previously described. IC₅₀ was expressed relative to the virus control using dose-response curves; and, in Graph Pad software for anti-viral activity curves analysis, a trendline that best suited the curve was selected and the correlating equation was used to calculate IC₅₀ values [69].

Anti-viral Activity Assays 449

The effect of the medicinal plants was tested by the anti-viral assays as following: 450

1. Direct Treatment of Virus-infected Cells (Anti-viral Activity) 451

To determine the effect of *H. perforatum*, *Echinacea* and *H.E.* on SARS CoV-2 infected 452
cells, the Vero E6 cells cultured on 96-well plates were infected with 100 TCID₅₀ of the virus 453
for 2hrs at 37°C, as described previously [14]. Then, treated with 1.56, 6.25, and 6.25 µg/mL 454
of *H. perforatum*, *Echinacea* and *H.E.*, respectively, at 37°C for 48h. The supernatant of the 455
cell samples was collected at every 12, 16, 24, 36, and 48hrs (3 wells per each time) [70] for 456
qRT-PCR [62]. According to above description, the relative mRNA expression levels of 457
SARS- CoV-2 RdRP gene was detected, the viral load of the cell samples was determined, 458
and the level of virus neutralization was assessed too. SARS-CoV-2-infected Vero E6 cells 459
were processed as controls. 460

2. Pre-treatment of Cells Prior to Virus Infection (Anti-viral Activity) 461

To investigate direct effects of *H. perforatum* on the virus, the Vero E6 cells cultured on 462
96-well plates were treated with 1.56, 6.25, and 6.25 µg/mL of *H. perforatum*, *Echinacea* and 463
H.E. solutions, respectively, at 37°C for 2hrs, and then washed 3 times with PBS solution 464
[14]. The cells were subsequently infected with SARS-CoV-2 (100 TCID₅₀) and cultured at 465
37°C for 48hrs. Also, the supernatant was collected every 12, 16, 24, 36, and 48hrs (3 wells 466
per each time) [70] for qPCR. We extend the observation till 48hrs to assess the extracts 467
efficacy after 36hrs post-infection. The relative mRNA expression levels of SARS CoV-2 468
RdRP gene, the viral load, and the level of virus neutralization were assessed with qRT-PCR 469
[62]. 470

3. Pre-Treatment of Virus Prior to Infection (Virucidal Activity) 471

To analyze the impact of *Echinacea* on cell whether to inhibit or not SARS-CoV-2 472
infection through neutralization binding receptor of the virus, 100 TCID₅₀ SARS-CoV-2 was 473

incubated with 1.56, 6.25, and 6.25 µg/mL *H. perforatum*, *Echinacea* and *H.E.*, respectively, 474
at 37°C for 2h [14]. The three extract-treated SARS-CoV-2 were subsequently used to infect 475
Vero E6 cells at 37°C for 48hrs. As described above, the relative mRNA expression levels of 476
SARS CoV-2 RdRP gene, viral load, and the level of virus neutralization were assessed with 477
qRT-PCR [62] of every 12, 16, 24, 36, and 48hrs supernatants cell samples [70]. During the 478
post infection cycle, the plates were analyzed by observing virus-induced CPE by light 479
microscope (Nikon-ECLIPSE-Ti), and plaque formation was determined by crystal violet 480
staining (C0775; Sigma-Aldrich), as previously described [47]. The supernatants were 481
collected and further used for qRT-PCR. 482

Quantitative Real-Time PCR (qRT-PCR) 483

Viral RNA was extracted from all samples collected directly from the 96 well plates 484
of the anti-viral assay, as previously described [62] using the QIAmp Viral RNA Mini Kit 485
(Qiagen, Germany) according to the manufacturer's instructions. Relative quantification of 486
the SARS-CoV-2 viral load was performed by one-step dual-target real time RT-PCR 487
(RealStar SARS-CoV-2 RT-PCR Kit 1.0, Altona Diagnostics, Germany) according to the 488
manufacturer's instructions using a 7500 Fast Real-Time PCR System (Applied Biosystems, 489
U.S.A.). The PCR detects a beta-coronavirus specific target (E-gene), a SARS-CoV- 490
specific target (RdRP-gene) and an internal control. The decrease in viral load was expressed 491
by comparing the cycle threshold (CT) values from each sample relative to the CT values of 492
the pretreatment inoculated sample (with the SARS-CoV-2 specific primers). The SARS- 493
CoV-2 titers were expressed as PFU equivalents per mL (PEq/mL) using a standard curve 494
(standard: serial dilutions of the viral stock) and choosing dilutions of the original sample 495
(10⁻¹ to 10⁻⁸) with CT values in the exponential phase. Each run included a positive viral 496
template control and no-template negative control. Each sample was tested in duplicate and 497
the mean is reported as PEq/mL. 498

499

Statistical analysis

500

Data was analyzed with one- or two-way ANOVA with a Tukey's test for multiple 501
comparisons. $P < 0.05$ and < 0.005 are considered statistically significant. All analyses were 502
performed with GraphPad Prism, version 8. 503

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504

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References	513
1. Fouchier R, Kuiken, T., Schutten, M. et al. Koch's postulates fulfilled for SARS virus. <i>Nature</i> 423, 240 (2003). DOI: 10.1038/423240a.	514 515
2. Van Der Hoek L PK, Jebbink MF, Vermeulen-Oost W, Berkhout RJ, Wolthers KC, et al. Identification of a new human coronavirus. <i>Nat Med.</i> 2004 Apr;10(4):368-73. doi: 10.1038/nm1024.	516 517
3. Vabret A DJ, Gouarin S, Petitjean J, Corbet S, Freymuth F. Detection of the new human coronavirus HKU1: a report of 6 cases. <i>Clin Infect Dis.</i> 2006;42(5):634-9. DOI: 10.1086/500136.	518 519
4. de Groot RJ, Baker SC, Baric RS, Brown CS, Drosten C, Enjuanes L, et al. Commentary: Middle east respiratory syndrome coronavirus (mers-cov): announcement of the coronavirus study group. <i>J Virol.</i> 2013;87(14):7790-2. doi: 10.1128/JVI.01244-13.	520 521 522
5. Giwa A, Desai A. Novel coronavirus COVID-19: an overview for emergency clinicians. <i>Emerg Med Pract.</i> 2020;22(2 Suppl 2):1-21.	523 524
6. Zhang L, Lin D, Sun X, Curth U, Drosten C, Sauerhering L, et al. Crystal structure of SARS-CoV-2 main protease provides a basis for design of improved α -ketoamide inhibitors. <i>Science.</i> 2020;368(6489):409-12. doi: 10.1126/science.abb3405.	525 526 527
7. Cui J, Li F, Shi Z-L. Origin and evolution of pathogenic coronaviruses. <i>Nat Rev Microbiol.</i> 2019;17(3):181-92. doi: 10.1038/s41579-018-0118-9.	528 529
8. Du Toit A. Outbreak of a novel coronavirus. <i>Nat Rev Microbiol.</i> 2020;18(3):123-. doi: 10.1038/s41579-020-0332-0.	530 531
9. Kirtipal N BS, Kang SG. From SARS to SARS-CoV-2, insights on structure, pathogenicity and immunity aspects of pandemic human coronaviruses. <i>Infect Genet Evol.</i> 2020 Nov; 85: 104502. doi: 10.1016/j.meegid.2020.104502.	532 533 534
10. Yang H YM, Ding Y, Liu Y, Lou Z, Zhou Z, et al. The crystal structures of severe acute respiratory syndrome virus main protease and its complex with an inhibitor. <i>Proc Natl Acad Sci.</i> 2003 Nov 11;100(23):13190-5. doi: 10.1073/pnas.1835675100.	535 536 537
11. Kupferschmidt K, Cohen J. Race to find COVID-19 treatments accelerates. <i>Science.</i> 2020;367(6485):1412. doi: 10.1126/science.367.6485.1412.	538 539
12. Lu H. Drug treatment options for the 2019-new coronavirus (2019-nCoV). <i>BioScience Trends.</i> 2020;14(1):69-71. doi: 10.5582/bst.2020.01020.	540 541
13. Dong L, Hu S, Gao J. Discovering drugs to treat coronavirus disease 2019 (COVID-19), <i>Drug Discov. Ther.</i> 14 (2020) 58–60. 2020.	542 543
14. Chen H, Muhammad I, Zhang Y, Ren Y, Zhang R, Huang X, et al. Antiviral Activity Against Infectious Bronchitis Virus and Bioactive Components of <i>Hypericum perforatum</i> L. <i>Front Pharmacol.</i> 2019;10:1272. doi: 10.3389/fphar.2019.01272.	544 545 546
15. Ng QX, Venkatanarayanan N, Ho CYX. Clinical use of <i>Hypericum perforatum</i> (St John's wort) in depression: a meta-analysis. <i>J Affect Disord.</i> 2017;210:211-21. doi: 10.1016/j.jad.2016.12.048.	547 548
16. Heydarian M, Jooyandeh H, Nasehi B, Noshad M. Characterization of <i>Hypericum perforatum</i> polysaccharides with antioxidant and antimicrobial activities: optimization based statistical modeling. <i>Int J Biol Macromol.</i> 2017;104:287-93. doi: 10.1016/j.ijbiomac.2017.06.049.	549 550 551
17. Porzel A, Farag MA, Mülbradt J, Wessjohann LA. Metabolite profiling and fingerprinting of <i>Hypericum</i> species: a comparison of MS and NMR metabolomics. <i>Metabolomics.</i> 2014;10(4):574-88. doi: 10.1007/s11306-013-0609-7.	552 553 554
18. Shamim Molla M, Azad AK, Al Hasib MAA, Hossain MM, Ahammed MS, Rana S, et al. A REVIEW ON ANTIVIRAL EFFECTS OF <i>NIGELLA SATIVA</i> L. 2019.	555 556
19. Weber N, Murray B, North J, Wood S. The antiviral agent hypericin has in vitro activity against HSV-1 through non-specific association with viral and cellular membranes. <i>Antivir Chem Chemother.</i> 1994;5(2):83-90. doi: 10.1177/095632029400500204.	557 558 559
20. Süntar I, Oyardı O, Akkol EK, Özçelik B. Antimicrobial effect of the extracts from <i>Hypericum perforatum</i> against oral bacteria and biofilm formation. <i>Pharm Biol.</i> 2016;54(6):1065-70. doi: 10.3109/13880209.2015.1102948.	560 561 562

21. Gibbons S, Ohlendorf B, Johnsen I. The genus *Hypericum*—a valuable resource of anti-staphylococcal leads. *Fitoterapia*. 2002;73(4):300-4. doi: 10.1016/s0367-326x(02)00082-5. 563
22. Marčetić MD, Milenković MT, Lakušić DV, Lakušić BS. Chemical Composition and antimicrobial activity of the essential oil and methanol extract of *Hypericum aegypticum* subsp. webbii (Spach) N. Robson. *Chem Biodivers*. 2016;13(4):427-36. doi: 10.1002/cbdv.201500119. 564
23. Saddiqe Z, Naeem I, Maimoona A. A review of the antibacterial activity of *Hypericum perforatum* L. *J Ethnopharmacol*. 2010;131(3):511-21. doi: 10.1016/j.jep.2010.07.034. 565
24. Schempp CM PK, Wittmer A, Schöpf E, Simon JC. Antibacterial activity of hyperforin from St John's wort, against multiresistant *Staphylococcus aureus* and gram-positive bacteria. *The Lancet*. 1999;353(9170):2129. doi: 10.1016/S0140-6736(99)00214-7. 566
25. Luettig B, Steinmüller C, Gifford G, Wagner H, Lohmann-Matthes M-L. Macrophage activation by the polysaccharide arabinogalactan isolated from plant cell cultures of *Echinacea purpurea*. *J Natl Cancer Inst*. 1989;81(9):669-75. doi: 10.1093/jnci/81.9.669. 567
26. Stimpel M, Proksch A, Wagner H, Lohmann-Matthes M. Macrophage activation and induction of macrophage cytotoxicity by purified polysaccharide fractions from the plant *Echinacea purpurea*. *Infect Immun*. 1984;46(3):845-9. doi: 10.1128/IAI.46.3.845-849.1984. 568
27. Goel V, Chang C, Slama JV, Barton R, Bauer R, Gahler R, et al. Alkylamides of *Echinacea purpurea* stimulate alveolar macrophage function in normal rats. *Int Immunopharmacol*. 2002;2(2-3):381-7. doi: 10.1016/S1567-5769(01)00163-1. 569
28. LaLone CA, Rizshsky L, Hammer KD, Wu L, Solco AK, Yum M, et al. Endogenous levels of *Echinacea* alkylamides and ketones are important contributors to the inhibition of prostaglandin E2 and nitric oxide production in cultured macrophages. *J Agric Food Chem*. 2009;57(19):8820-30. doi: 10.1021/jf901202y. 570
29. Rana J, Chandra A. Need for analytical methods and fingerprinting: total quality control of phytomedicine *Echinacea*. ACS Publications; 2006. 571
30. Sasagawa M, Cech NB, Gray DE, Elmer GW, Wenner CA. *Echinacea* alkylamides inhibit interleukin-2 production by Jurkat T cells. *Int Immunopharmacol*. 2006;6(7):1214-21. doi: 10.1016/j.intimp.2006.02.003. 572
31. Speroni E, Govoni P, Guizzardi S, Renzulli C, Guerra M. Anti-inflammatory and cicatrizing activity of *Echinacea pallida* Nutt. root extract. *J Ethnopharmacol*. 2002;79(2):265-72. doi: 10.1016/S0378-8741(01)00391-9. 573
32. Wills R, Stuart D. Alkylamide and cichoric acid levels in *Echinacea purpurea* grown in Australia. *Food Chem*. 1999;67(4):385-8. doi: 10.1016/S0308-8146(99)00129-6. 574
33. Cundell DR, Matrone MA, Ratajczak P, Pierce Jr JD. The effect of aerial parts of *Echinacea* on the circulating white cell levels and selected immune functions of the aging male Sprague–Dawley rat. *Int Immunopharmacol*. 2003;3(7):1041-8. doi: 10.1016/S1567-5769(03)00114-0. 575
34. Goel V, Chang C, Slama JV, Barton R, Bauer R, Gahler R, et al. *Echinacea* stimulates macrophage function in the lung and spleen of normal rats. *J Nutr Biochem*. 2002;13(8):487-92. doi: 10.1016/s0955-2863(02)00190-0. 576
35. Brush J, Mendenhall E, Guggenheim A, Chan T, Connelly E, Soumyanath A, et al. The effect of *Echinacea purpurea*, *Astragalus membranaceus* and *Glycyrrhiza glabra* on CD69 expression and immune cell activation in humans. *PUBDB*. 2006;20(8):687-95. doi: 10.1002/ptr.1938. 577
36. Currier N, Miller S. Natural killer cells from aging mice treated with extracts from *Echinacea purpurea* are quantitatively and functionally rejuvenated. *Exp Gerontol*. 2000;35(5):627-39. doi: 10.1016/s0531-5565(00)00106-6. 578
37. Fast DJ BJ, Scholten JD, Mulder T, Rana J. *Echinacea purpurea* root extract inhibits TNF release in response to Pam3Csk4 in a phosphatidylinositol-3-kinase dependent manner. *Cell Immunol*. 2015;297(2):94-9. doi: 10.1016/j.cellimm.2015.07.003. 579
38. Gertsch J, Schoop R, Kuenzle U, Suter A. *Echinacea* alkylamides modulate TNF- α gene expression via cannabinoid receptor CB2 and multiple signal transduction pathways. *FEBS Lett*. 2004;577(3):563-9. doi: 10.1016/j.febslet.2004.10.064. 580

39. Cech NB, Kandhi V, Davis JM, Hamilton A, Eads D, Laster SM. Echinacea and its alkylamides: Effects on the influenza A-induced secretion of cytokines, chemokines, and PGE2 from RAW 264.7 macrophage-like cells. *Int Immunopharmacol.* 2010;10(10):1268-78. doi: 10.1016/j.intimp.2010.07.009.
40. Matthias A, Banbury L, Stevenson L, Bone K, Leach D, Lehmann R. Alkylamides from Echinacea modulate induced immune responses in macrophages. *Immunol Invest.* 2007;36(2):117-30. doi: 10.1080/08820130600745786.
41. Pleschka S, Stein M, Schoop R, Hudson JB. Anti-viral properties and mode of action of standardized Echinacea purpurea extract against highly pathogenic avian influenza virus (H5N1, H7N7) and swine-origin H1N1 (S-OIV). *Viol J.* 2009;6(1):1-9. doi: 10.1186/1743-422X-6-197.
42. V'kovski P, Kratzel A, Steiner S, Stalder H, Thiel V. Coronavirus biology and replication: implications for SARS-CoV-2. *Nat Rev Microbiol.* 2020:1-16. doi: 10.1038/s41579-020-00468-6.
43. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, et al. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell.* 2020. doi: 10.1016/j.cell.2020.02.052.
44. Ou X, Liu Y, Lei X, Li P, Mi D, Ren L, et al. Characterization of spike glycoprotein of SARS-CoV-2 on virus entry and its immune cross-reactivity with SARS-CoV. *Nat Commun.* 2020;11(1):1-12. doi: 10.1038/s41467-020-15562-9.
45. Polack FP, Thomas SJ, Kitchin N, Absalon J, Gurtman A, Lockhart S, et al. Safety and efficacy of the BNT162b2 mRNA covid-19 vaccine. *N Engl J Med.* 2020. doi: 10.1056/NEJMoa2034577.
46. Jeon S, Ko M, Lee J, Choi I, Byun SY, Park S, et al. Identification of antiviral drug candidates against SARS-CoV-2 from FDA-approved drugs. *Antimicrob Agents Chemother.* 2020. doi: 10.1128/AAC.00819-20.
47. Sui X, Yin J, Ren X. Antiviral effect of diammonium glycyrrhizinate and lithium chloride on cell infection by pseudorabies herpesvirus. *Antivir Res.* 2010;85(2):346-53. doi: 10.1016/j.antiviral.2009.10.014.
48. Prince AM, Pascual D, Meruelo D, Liebes L, Mazur Y, Dubovi E, et al. Strategies for evaluation of enveloped virus inactivation in red cell concentrates using hypericin. *Photochem Photobiol.* 2000;71(2):188-95. doi: 10.1562/0031-8655(2000)071<0188:sfeoev>2.0.co;2.
49. Jacobson JM, Feinman L, Liebes L, Ostrow N, Koslowski V, Tobia A, et al. Pharmacokinetics, safety, and antiviral effects of hypericin, a derivative of St. John's wort plant, in patients with chronic hepatitis C virus infection. *Antimicrob Agents Chemother.* 2001;45(2):517-24. doi: 10.1128/AAC.45.2.517-524.2001.
50. Barnes J, Anderson LA, Phillipson JD. St John's wort (*Hypericum perforatum* L.): a review of its chemistry, pharmacology and clinical properties. *J Pharm Pharmacol.* 2001;53(5):583-600. doi: 10.1211/0022357011775910.
51. Birt DF, Widrechner MP, Hammer KD, Hillwig ML, Wei J, Kraus GA, et al. Hypericum in infection: Identification of anti-viral and anti-inflammatory constituents. *Pharm Biol.* 2009;47(8):774-82.
52. Xiuying P, Jianping L, Ruofeng S, Liye Z, Xuehong W, Yan L. Therapeutic efficacy of *Hypericum perforatum* L. extract for mice infected with an influenza A virus. *Can J Physiol Pharmacol.* 2012;90(2):123-30. doi: 10.1139/y11-111.
53. Jawad M, Schoop R, Suter A, Klein P, Eccles R. Safety and efficacy profile of Echinacea purpurea to prevent common cold episodes: a randomized, double-blind, placebo-controlled trial. *Evid-Based Complementary Altern Med.* 2012. doi: 10.1155/2012/841315.
54. Rauš K, Pleschka S, Klein P, Schoop R, Fisher P. Effect of an Echinacea-based hot drink versus oseltamivir in influenza treatment: a randomized, double-blind, double-dummy, multicenter, noninferiority clinical trial. *Curr Ther Res.* 2015;77:66-72. doi: 10.1016/j.curtheres.2015.04.001.
55. Schapowal A. Efficacy and safety of Echinaforce® in respiratory tract infections. *Wien Med Wochenschr Suppl.* 2013;163(3-4):102-5. doi: 10.1007/s10354-012-0166-0.

56. Schapowal A, Klein P, Johnston SL. Echinacea reduces the risk of recurrent respiratory tract infections and complications: a meta-analysis of randomized controlled trials. *Adv Ther.* 2015;32(3):187-200. doi: 10.1007/s12325-015-0194-4. 664 665 666
57. Sharma M, Anderson SA, Schoop R, Hudson JB. Induction of multiple pro-inflammatory cytokines by respiratory viruses and reversal by standardized Echinacea, a potent antiviral herbal extract. *Antivir Res.* 2009;83(2):165-70. doi: 10.1016/j.antiviral.2009.04.009. 667 668 669
58. Kindler E, Jónsdóttir HR, Muth D, Hamming OJ, Hartmann R, Rodriguez R, et al. Efficient replication of the novel human betacoronavirus EMC on primary human epithelium highlights its zoonotic potential. *MBio.* 2013;4(1). doi: 10.1128/mBio.00611-12. 670 671 672
59. Sharma M, Schoop R, Hudson J. The efficacy of Echinacea in a 3-D tissue model of human airway epithelium. *Phytother Res.* 2010;24(6):900-4. doi: 10.1002/ptr.3051. 673 674
60. Signer J, Jonsdottir HR, Albrich WC, Strasser M, Züst R, Ryter S, et al. In vitro virucidal activity of Echinaforce®, an Echinacea purpurea preparation, against coronaviruses, including common cold coronavirus 229E and SARS-CoV-2. *Phytother Res.* 2020;17(1):136. doi: 10.1186/s12985-020-01401-2. 675 676 677 678
61. Andreania J, Le Bideau M, Duflota I, Jardota P, Rollanda C, Boxbergera M, et al. In vitro testing of hydroxychloroquine and azithromycin on SARS-CoV-2 shows 1 synergistic effect 2. *lung.* 2020;21:22. doi: 10.1016/j.micpath.2020.104228. 679 680 681
62. Azhar EI, Hindawi SI, El-Kafrawy SA, Hassan AM, Tolah AM, Alandijany TA, et al. Amotosalen and ultraviolet A light treatment efficiently inactivates severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in human plasma. *Vox Sang.* 2020. doi: 10.1111/vox.13043. 682 683 684
63. Bauer R. Chemistry, analysis and immunological investigations of Echinacea phytopharmaceuticals. *Immunomodulatory agents from plants: Springer;* 1999. p. 41-88. 685 686
64. Diraz E, Karaman S, Koca N, editors. Fatty acid and essential oil composition of Echinacea purpurea (L.) Moench, growing in Kahramanmaras–Turkey. *International conference on environmental and biological sciences (ICEBS'2012), Bangkok;* 2012. 687 688 689
65. Erdelmeier C. Hyperforin, possibly the major non-nitrogenous secondary metabolite of *Hypericum perforatum* L. *Pharmacopsychiatry.* 1998;31(S 1):2-6. doi: 10.1055/s-2007-979339. 690 691
66. Nahrstedt A, Butterweck V. Biologically active and other chemical constituents of the herb of *Hypericum perforatum* L. *Pharmacopsychiatry.* 1997;30(S 2):129-34. doi: 10.1055/s-2007-979533. 692 693
67. Kuppusamy P, Yusoff MM, Parine NR, Govindan N. Evaluation of in-vitro antioxidant and antibacterial properties of *Commelina nudiflora* L. extracts prepared by different polar solvents. *Saudi J Biol Sci.* 2015;22(3):293-301. doi: 10.1016/j.sjbs.2014.09.016. 694 695 696
68. Lyles JT, Kim A, Nelson K, Bullard-Roberts AL, Hajdari A, Mustafa B, et al. The chemical and antibacterial evaluation of St. John's Wort oil macerates used in Kosovar traditional medicine. *Front Microbiol.* 2017;8:1639. doi: 10.3389/fmicb.2017.01639. 697 698 699
69. Pourianfar HR, Poh CL, Fecondo J, Grollo L. In vitro evaluation of the antiviral activity of heparan sulfate mimetic compounds against Enterovirus 71. *Virus Res.* 2012;169(1):22-9. doi: 10.1016/j.virusres.2012.06.025. PubMed PMID: 22771616. 700 701 702
70. Brahim Belhaouari D, Fontanini A, Baudoin J-P, Haddad G, Le Bideau M, Bou Khalil JY, et al. The strengths of scanning electron microscopy in deciphering SARS-CoV-2 infectious cycle. *Front Microbiol.* 2020;11:2014. 703 704 705 706

Figure 1. Cell viability of Vero E6 was determined by MTT assay. The survival rate of cells treated with different concentrations of *H. perforatum*, *Echinacea* and *H.E.* ranging from 100 to 0.78 µg/mL. The percentage cell viability was calculated relatively to cell control wells. More than 50% cell survival rate was considered to be the maximum non-toxic concentration of H, E and H.E. Statistical analysis showed that data were significant with $p < 0.005$ (one-way ANOVA) of three independent experiments.

Figure 2. Dose-response curve analysis by Graph pad software for *H. perforatum*, *Echinacea* and *H.E.* activities. The black curve represents inhibition of SARS-CoV-2 infection (%) and the red dots represent log concentration ($\mu\text{g/ml}$).

(A) Direct Treatment of Virus-infected Cells

(B) Pre-treatment of Cells prior to Virus Infection

(C) Pre-treatment of Virus Prior to Infection

Figure 3. The inhibitory effect of *H. perforatum*, *Echinacea*, and *H.E.* on mRNA expression levels of SARS-CoV-2 RdRP gene in Vero E6 cells was evaluated by qRT-PCR. The impact of plant materials was detected by the three experimental designs: Direct Treatment of Virus-infected Cells (A), Pre-treatment of Cells Prior to Virus Infection (B), and Virucidal (C) assays; treated and infected cells were included as negative and positive controls, respectively. So, 100 TCID₅₀ of the virus was added with the certain concentrations of the plant materials when the cells at 80% confluence were complete. The viral inhibition percentage calculated relative to virus control wells, and representatives of two independent experiments performed in triplicate are shown. Statistical analysis showed that data were significant with $p < 0.05$ and $p < 0.005$ (one- & two-ways ANOVA).

Figure 4. The effect of *H. perforatum*, *Echinacea*, and *H.E.* on viral load of SARS-CoV-2 infection in Vero E6 cells was evaluated by qRT-PCR. The impact of plant materials was detected by the three experimental designs: Direct Treatment of Virus-infected Cells (A), Pre-treatment of Cells Prior to Virus Infection (B), and Virucidal (C) assays; treated and infected cells were included as negative and positive controls (PC), respectively. So, 100 TCID₅₀ of the virus was added with the certain concentrations of the plant materials when the cells at 80% confluence were complete. The effect of the plant materials was observed clearly with reduction in viral load at 1.56, 6.25, and 6.25 µg/ml, respectively. The viral load was calculated relatively to virus control wells, and representatives of two independent experiments performed in triplicate are shown. Statistical analysis showed that data were significant with $p < 0.005$ (one- & two-ways ANOVA).

A				B				C			
	24H	36H	48H		24H	36H	48H		24H	36H	48H
H				H				H
E				E				E
HE				HE				HE

Figure 5. The impact of *H. perforatum*, *Echinacea* and *H.E.* on SARS-CoV-2 infected-Vero E6 cells by antiviral activity essays. The cell viability and CPE through SARS-CoV-2 infectious cycle, from 24 to 48 hours post infection, were visualized by photographed images with a light microscope (magnification, x100) (orange squares) and by the crystal violet assay (blue circles). The images were captured when the CPE started on the positive control at 24h

Figure 6. The effect of *H. perforatum*, *Echinacea*, and *H.E.* on viral load of SARS-CoV-2 infection in Vero E6 cells was evaluated by qRT-PCR. The impact of plant materials was detected by the three experimental designs: Direct Treatment of Virus-infected Cells (A), Pre-treatment of Cells Prior to Virus Infection (B), Virucidal (C) assays, and positive control (PC). The effect of the plant materials was observed clearly with reduction in viral load at 1.56 µg/ml for *H. perforatum*, then for *H.E.* at 6.25 µg/ml, and finally for *Echinacea* at 6.25 µg/ml.