Composition of naturally occurring compounds decreases activity of Omicron and SARS-CoV-2 RdRp complex

ANNA GOC, MATTHIAS RATH and
ALEKSANDRA NIEDZWIECKI* 🅾

Dr. Rath Research Institute, 5941 Optical Ct., San Jose, CA 95138, USA

ABSTRACT

Naturally-occurring compounds are acknowledged for their broad antiviral efficacy. Little is however known about their mutual cooperation. Here, we evaluated in vitro efficacy of the defined mixture of agents against the RdRp complex of the original SARS-CoV-2 and Omicron variant. This composition of vitamin C, N-acetylcysteine, resveratrol, theaflavin, curcumin, quercetin, naringenin, baicalin, and broccoli extract showed to inhibit activity of RdRp/nsp7/nsp8 both these variants. In vitro exposure of recombinant RdRp complex to individual compounds of this composition pointed to quercetin as the driving inhibitory compound. The outcome of this study supports the motion of antiviral efficacy of natural compounds against SARS-CoV-2 and Omicron and implies that their reciprocal or mutual interaction may augment antiviral action through simultaneous effect on different mechanisms. Consequently, this makes it more difficult for an infectious agent to evade all these mechanisms at the same time. Considering the urgency in finding effective prevention, but also side-effects free treatment of COVID-19 our results call for clinical affirmation of the benefits of this micronutrient combination in both preventive and therapeutic aspects. Whether observed effects can be achieved, by concentrations of the active agents used in these in vitro experiments, in in vivo or clinical setting warrants further study.

*Corresponding author. Dr. Rath Research Institute BV, 5941 Optical Ct., San Jose, CA 95138, USA. Tel.:+ 1 408-567-5000. E-mail: author@drrath.com

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INTRODUCTION

Various preventive and therapeutic approaches to curb the COVID-19 pandemic have been continuously challenged by the emergence of various variants of SARS-CoV-2, including the most recent Omicron variant.

Human coronaviruses (CoVs) are made up of structural proteins, which include the spike (S) protein, the nucleocapsid (N), matrix (M), and envelope (E) proteins. In addition, the viruses also contain non-structural proteins (nsps) such as nsp3 and nsp5, as well as RNA-Dependent RNA Polymerase (RdRp, nsp12) [1].

RdRp of SARS-CoV-2 plays a crucial role in its life cycle and is the protein that has been universally conserved among RNA viruses [2]. RdRps are multi-domain proteins that catalyze RNA-template dependent formation of phosphodiester bonds between ribonucleotides in the presence of divalent metal ions [3]. Upon infecting human cells, the CoV positive-strand RNA is translated to produce a long polyprotein that is cleaved into several nonstructural proteins (nsps), which are required for viral replication and gene expression [4]. Among these, nsp12, a catalytic subunit of RdRp, in complex with accessory subunits nsp7 and nsp8, plays a central role. Since RdRp contains intrinsically disordered regions (IDRs) that undergo large context-dependent conformational changes, it has been suggested that RdRp activity can be modulated, positively or negatively, by various factors that control the folding of the enzyme [5].

RdRp activity has been a target in development of several inhibitors that are used to treat a wide range of RNA viruses including Ebola, the human immunodeficiency virus (HIV), and the Zika virus [6]. Although these inhibitors are based on different mechanisms, one of the ways by which they can inhibit viral RNA replication is by replacing ATP in the newly synthesized viral RNA strand and, as such, terminate the RNA synthesis process and prevent further replication of the virus.

With the outbreak of COVID-19 various already existing anti-viral drugs, including nucleoside analogs that inhibited RNA synthesis in other viruses, were re-purposed against the SARS-CoV-2 [7]. In addition to these drugs, corticosteroids and interferon have also been tested for their therapeutic efficacy against SARS-CoV-2 [8, 9]. Various natural compounds including polyphenols also have been evaluated against RdRp activity through *in silico* approaches [10].

Recent emergence of the Omicron variant with its multiple mutations in the spike protein has turned attention towards less mutation prone viral components, including RdRp. Newly detected point mutations in RdRp of the Omicron variant targeted by antiviral drugs (i.e., remdesivir, molnupiravir) raised concern of decreased effectiveness of these therapeutics against future variants [3, 11]. However, still inhibition of RdRp activity and, as such, viral replication remains an attractive target.

Our earlier study revealed that a combination of ascorbic acid, N-acetylcysteine (NAC) and various polyphenols could simultaneously affect key cellular mechanisms involved in SARS-CoV-2 infectivity [12]. These include inhibition of SARS-CoV-2-RBD binding to the ACE2 receptor, decreased cathepsin L and furin activity, decreased number of ACE2 receptors on human lung epithelial cells, and lower enzymatic activity of RdRp/nsp12. We also showed that this composition was effective in curbing cellular entry of SARS-CoV-2 virions and its variants: Alpha, Beta, Delta, Gamma, and Mu [13].

Taking into account multi-target efficacy of this composition combined with safety, low cost and wide availability of these natural compounds, we conducted further investigation of its effects on inhibition of RdRp complex activity originating from Omicron and SARS-CoV-2.

MATERIAL AND METHODS

Cell line and viral proteins

A549 obtained from American Type Culture Collection (Manassas, VA) was purchased from GenScript (Piscataway, NJ). Omicron RdRp and SARS-CoV-2 RdRp (as a complex of nsp12/nsp7/nsp8) were from BPS Bioscience (San Diego, CA). All other compounds were from Sigma (St. Louis, MO) except for broccoli extract that was from Bulk Supplements (Henderson, NV).

Plant-derived compositions

The combinations of natural compounds tested in this study were: curcumin, quercetin, naringenin, baicalin, theaflavin 3′3″ digallate, ascorbic acid, (NAC), resveratrol, and broccoli extract. Stock solutions of these agents were prepared in DMSO at 50 mg ml^{-1} and kept at $-20\,°C$ until analysis. For the experiments, the stock solution was diluted with $1 \times$ PBS or a buffer recommended by the manufacturer of the utilized kit (enzyme activity assays) to final concentrations indicated in the Figures. Final DMSO concentrations used did not excided 0.05 and were in the allowed range of 1.0%, which is a maximum allowed concentration of DMSO by the manufacturer of the kit. Our selection of these test compounds was based on obtained results from extensive screening experiments targeting crucial steps and aspects in SARS-CoV-2 infection that revealed the best performing agents.

In vitro RdRp activity assay

In vitro RdRp activity was evaluated using an RNA Polymerase Assay Kit (ProFoldin, Hudson, MA) according to the manufacturer's protocol. Briefly, 0.5 µl of $50 \times$ recombinant either Omicron RdRp or SARS-CoV-2 RdRp was incubated with 2.5 µl of $50 \times$ buffer, 20 µl of water, and 1.0 µl of individual compound or a mixture of them at $0–100\,µg\ ml^{-1}$ concentrations for 15 min at RT, followed by the addition of the master mix containing 0.5 µl of $50 \times$ NTPs and 0.5 µl of $50 \times$ template (as a single-stranded polyribonucleotide). The reaction (25 µl) was incubated for 2 h at $34\,°C$ and then stopped by the addition of 65 µl of $10 \times$ fluorescence dye, and the fluorescence signal was recorded in 10 min at extension/ emission $= 488/535$ nm using a fluorescence spectrometer (Tecan, Group Ltd., Switzerland). Negative control contained of 100% dead cells. Results are expressed as a % of control without compound addition (mean $+/-$ SD, $n = 5$).

Cytotoxicity

Cells viability was assessed using MTT assay according to the manufacturer's protocol. Briefly, human A549 cells was plated in 96-well plates at 1×10^4 cells per well in the DMEM containing 10% FBS. After 24 h, the medium was replaced with the same medium supplemented with the test compounds. After 24 h of treatment, cells viability was measured at 570 nm, using an ELISA reader (Molecular Device, Spectra Max 340). Results are expressed as a % of control without compound addition (mean $+/-$ SD, $n = 8$).

Statistical analysis

Data for all experiments are presented as an average value and standard deviation from at least three independent experiments. Comparison between different samples was done by a two-tailed *T*-test using the Microsoft Office Excel program. Differences between samples were considered significant at *P* values less than 0.05.

Ethics

No human subjects were involved in this study. Therefore IRB ethical approval and informed consent are not applicable.

Table 1. Effect of each compound of the test formula on activity of Omicron and SARS-CoV-2 RdRp complex

Test agent (0.1 mg ml^{-1})	Inhibition of RdRp activity (% control ± SD)	
	Omicron nsp12/nsp7/nsp8	SARS-CoV-2 nsp12/nsp7/nsp8
Curcumin	88.1±4.2*	89.7±5.1*
Theaflavin 3′3 digallate	90.5±5.2*	92.1±6.4*
Quercetin	**99.9±3.4***	**99.8±3.5***
Baicalin	64.9±4.4*	50.3±5.4*
Resveratrol	55.2±6.5*	55.4±5.6*
Naringinin	61.2±5.1*	57.9±5.7*
NAC	15.3±4.2^	25.3±4.8^
Broccoli extract	17.8±3.9^	23.9±7.9^
Ascorbic acid	37.1±2.1^	31.7±4.1^

0.1 mg ml^{-1} of test agent was first incubated with the mix containing either Omicron or SARS-CoV-2 RdRp complex for 15 min at RT and then supplemented with NTPs and template, and further incubated for an additional 2 h at 34 °C. Plates were then developed with 1 × fluorescence dye for up to 10 min. Fluorescence was measured at Ex/Em = 480/535 nm; ^ $P \leq 0.01$, * $P \leq 0.001$.

RESULTS

The results in Table 1 show the effects of individual compounds applied at 0.1 mg ml^{-1} concentrations on activity of Omicron and SARS-CoV-2 RdRp complex (nsp12/nsp7/nsp8).

All test compounds inhibited activity of both Omicron and SARS-CoV-2 RdRp complex, but at a different degree. The most effective RdRp inhibition ranging from 88.1% to 99.9% was obtained with quercetin, theaflavin3′3″ digallate, and curcumin. Their inhibitory effects were comparable for Omicron and SARS-CoV-2 RdRp complex. Baicalin, naringenin, and resveratrol also showed RdRp inhibition by 64.9%, 55.2% and 61.2%, respectively for Omicron, and 50.3%, 55.4% and 57.9%, respectively for SARS-CoV-2 RdRp complex. The two most effective inhibitory compounds,

quercetin and theaflavin 3′3″ digallate, and the mixture of all test compounds were evaluated further.

In order to exclude cytotoxic effects of test compounds and their mixture, we evaluated viability of ACE2 over-expressing human alveolar epithelial cells (A549) exposed to increasing concentrations of quercetin and theaflavin 3′3″ digallate, as the most effective ingredients against RdRp complex, and the mixture of all compounds.

The results in Fig. 1 show that both quercetin and theaflavin 3′3″ digallate applied up to 25 μg ml^{-1} concentrations did not affect cells viability. In the presence of all test compounds combined together, up to 10 μg ml^{-1} of the cells remained viable, and at 25 μg ml^{-1} the cells viability decreased by 35%. Therefore, effects of both the mixture, and quercetin and theaflavin 3′3″ digallate on RdRp activity were evaluated at 10 μg ml^{-1}.

Figure 2A and 2B present the effects of quercetin and theaflavin 3′3″ digallate applied at 10 μg ml^{-1} and 25 μg ml^{-1} concentrations on enzymatic activity of Omicron and SARS-CoV-2 RdRp complex, respectively. Both compounds showed dose-dependent inhibitory effects, and quercetin at 10 μg ml^{-1} decreased RdRp activity by 43% and 44% for Omicron and SARS-CoV-2, respectively. Theaflavin 3′3″ digallate at 10 μg ml^{-1} was less effective and could lower RdRp activity by 21% and 22% for Omicron and SARS-CoV-2, respectively.

The results in Fig. 2C compare the efficacy of mixture used at non-toxic 10 μg ml^{-1} concentration on Omicron and SARS-CoV-2 RdRp activities. In the presence of MixV the RdRp activity decreased by 41% for SARS-CoV-2 RdRp complex and 42% inhibition for its Omicron variant.

DISCUSSION

This study further expands our previous investigations on the efficacy of natural compounds on key cellular mechanisms involved in the infectivity of SARS-CoV-2 and its variants. Here we show that in addition to simultaneous
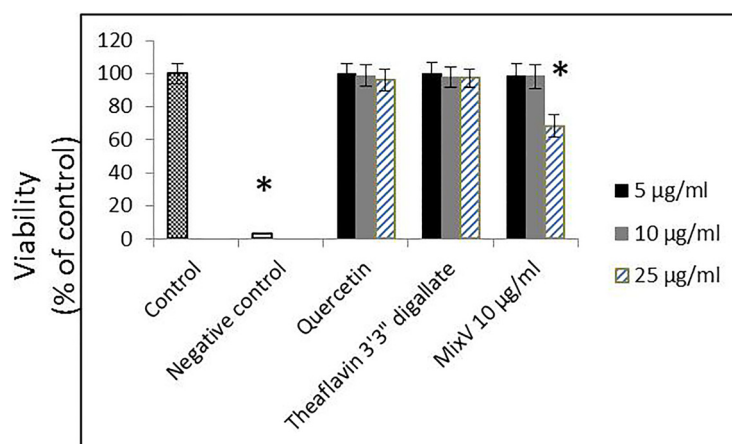

Fig. 1. Effects of selected individual compounds and MixV on survival of A549 cells. Cellular viability upon treatment with either individual compound or MixV composition at designated concentrations for 24 h minutes at 37 °C. Control – 0.05% DMSO, negative control – 100% dead cells; * $P \leq 0.001$
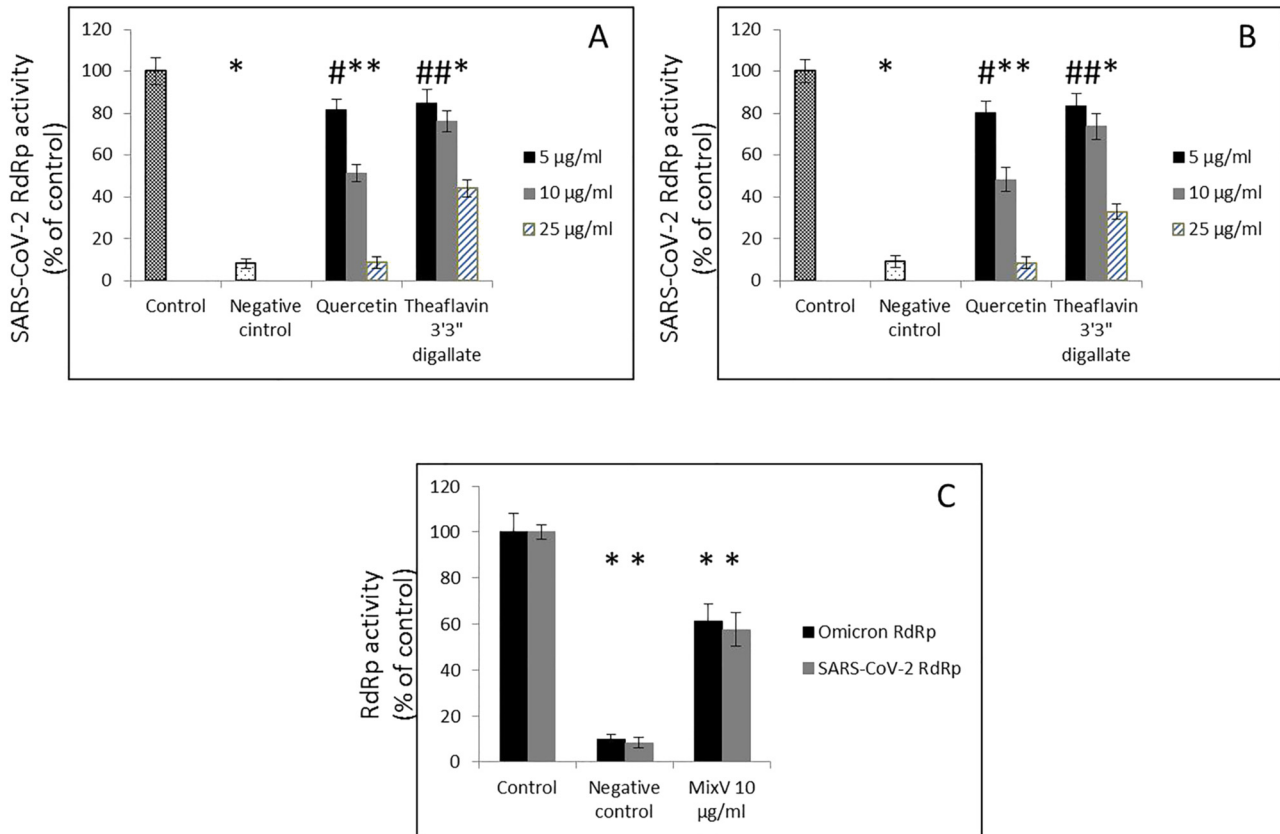
Fig. 2. Effects of selected individual compounds and MixV on viral RdRp complex. Dose-dependent effects of the most inhibitory compounds of MixV composition on activity of Omicron nsp12/nsp7/nsp8 (A) and SARS-CoV-2 nsp12/nsp7/nsp8 (B). Effects of MixV composition alone on activity of Omicron nsp12/nsp7/nsp8 and SARS-CoV-2 nsp12/nsp7/nsp8, respectively (C). Purified recombinant RdRp complex enzyme was incubated with either individual compound or MixV composition at designated concentrations for 15 min at RT. After addition of reaction mix composed of NTPs and RNA template incubation was carry out for 2h at 34 °C. Signal was measured in 10 min at extension/emission = 488/535 nm with spectrofluorometer. Control – 0.05% DMSO, negative control – 100% of inhibited enzyme; # $P \leq 0.05$, * $P \leq 0.001$

inhibition of cellular internalization of SARS-CoV-2 and its variants (Alpha, Beta, Gamma, Delta, Mu) demonstrated earlier using the mixture of test compounds used in this study [13], the inhibitory effects of these natural compounds also expands to RdRp activity.

Among the individual compounds tested in the study both quercetin and theaflavin 3′3 digallate had the highest, almost 100%, inhibitory effects on RdRp complex when applied at 0.1 mg ml^{-1}. Quercetin had 90% inhibitory effect also at its lower concentration of 25 µg ml^{-1}. Interestingly, in addition to RdRp activity, theaflavin 3′3″ digallate has shown to be effective in inhibiting RBD binding by 100%, however quercetin had by 22.3% lower RDB binding inhibitory effect [14].

These compounds are a part of a nutritional complex – MixV – where anti-SARS-CoV-2 efficacy was evaluated in our earlier studies and shown to be effective in simultaneous control of several cellular mechanisms involved in this viral infectivity. Among these, there was an indication of MixV's inhibitory effect on RdRp activity when using a recombinant enzyme (nsp12) and whole cell lysates [12]. Our present results show that inhibitory efficacy of this mixture expands

to RdRp complex (nsp12/nsp7/nsp8) of both SARS-CoV-2 and Omicron variants, which would signal a possibility of application of micronutrients in controlling RdRp activity across a wider spectrum of viral species.

In order to achieve the multi-target efficacy, such as the one observed with MixV, the selection of components is important as each of them shows distinct beneficial effects in select cellular mechanisms. As an example, while theaflavin 3′3″ digallate could inhibit in nearly 100% both RBD binding and RdRp activity in SARS-CoV-2 and Omicron variants, quercetin had lower efficacy in inhibiting RBD binding but was 100% effective as an RdRp inhibitor [14]. Ascorbic acid was not significantly affecting RdRp activity and RBD binding [13], however it was effective in decreasing ACE2 receptor expression, decreasing inflammation [15], and as well showed clinical efficacy in advanced COVID-19 [16]. The advantage of a multi-target approach over a focus on a single metabolic mechanism includes simultaneous impact on the complexity of viral metabolism and is less prone to mutational changes, which could translate to an overall better efficacy.

Inhibition of RdRp complex activity in two coronavirus variants by MixV confirms pleiotropic metabolic efficacy of
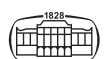
the natural compounds, although the precise mechanisms of this inhibition were not addressed in this study. Since RdRp is rather conservative among coronavirus species, this nutrient efficacy may expand to RdRp inhibition in other coronavirus species, which deserve further studies.

Naturally occurring compounds, their derivatives, and metabolites are widely known for their antioxidant, antiviral, antimicrobial, and anti-inflammatory properties. Several studies highlight, for example, the antiviral potential of quercetin against the SARS virus, due to its ability to interfere with various stages of the coronavirus entry and replication cycle such as PLpro, 3CLpro, and NTPase/helicase. Molecular docking analysis also found that quercetin binds to its spike protein, RdRp, and PLpro enzymes. There is also evidence that quercetin in combination with vitamins C and D, may exert a synergistic antiviral action and thus serve as either an alternative or additional therapeutic and preventive option due to interrelating antiviral and immune-modulatory properties. Quercetin also demonstrated *in vitro* dose-dependent activity against herpes simplex virus HSV-1 and HSV-2, inhibitory anti-cytopathic effects caused by various serotypes of rhinovirus, echovirus, coxsackievirus, and poliovirus type 1 Sabin [17]. There is also a tremendous amount of literature supporting the antiviral properties of baicalin, in both *in vitro* and *in vivo* experiments. Baicalin exhibited a significant antiviral activity with $IC_{50} = 14.9\,\mu g\,ml^{-1} \pm 0.07$ against intracellular DENV-2 intracellular replicon by targeting non-structural proteins of the virus. This observation corroborated the data obtained in the time-of-drug-addition studies. Baicalin also exerts its anti-influenza virus activity by modulating viral protein NS1, resulting in up-regulation of IFN-induced antiviral signaling, and a decrease in PI3K/Akt signaling in cells [18]. Resveratrol as well is a potent polyphenolic compound that has been extensively studied in the amelioration of viral infections both in vitro and in vivo. Its antioxidant effect is mainly elicited through inhibition of important gene pathways like the NF-$\kappa\beta$ pathway, while its antiviral effects are associated with inhibitions of viral replication, protein synthesis, gene expression, and nucleic acid synthesis [19]. The beneficial effects of tea have been mostly attributed to its catechin content. However, black tea derived from the leaves of the *Camellia sinensis* plant, is rich in theaflavin polyphenols, in particular theaflavin (TF1), theaflavin-3-monogallate (TF2A), theaflavin-3′-monogallate (TF2B), and theaflavin-3,3′-digallate (TF3). Results from experiments on Vero and A549 cells used to evaluate the effect of purified individual black tea theaflavins as anti-herpes simplex virus 1 agents showed that TF1, TF2, and TF3 exhibit potent, dose-dependent anti-HSV-1 effect, with TF3 being the most efficient in both Vero and A549 cells. A concentration of 50 μM TF3 and above was sufficient to inhibit >99% of the production of HSV-1 viral particles. Based on these results, the anti-herpes viral activity of theaflavins were in the order of TF3 > TF2 > TF1 [20]. In aspect of SARS-CoV-2, especially the role of zinc as an anti-viral agent has been acknowledged. As zinc is essential to preserve natural tissue barriers such as the respiratory epithelium, preventing pathogen entry, for a balanced function of the immune system and the redox system, zinc deficiency can probably be added to the factors predisposing individuals to infection and detrimental progression of COVID-19. Antiviral effects of zinc have been reported as well, e.g. against coronaviridae, picornavirus, papilloma virus, metapneumovirus, rhinovirus, herpes simplex virus, varicella-zoster virus, respiratory syncytial virus, human immunodeficiency virus (HIV), and the hepatitis C virus; and it was suggested that zinc can prevent fusion with the host membrane, decrease the viral polymerase function, impair protein translation and processing, block viral particle release, and destabilize the viral envelope. Low-dose zinc supplementation together with small concentrations of the zinc ionophores pyrithione or hinokitol decreased RNA synthesis in influenza, poliovirus, picornavirus, the equine arteritis virus, and SARS-CoV-1 by directly inhibiting the RNA-dependent RNA polymerase of the virus [21]. Ordonez et al. evaluated the antiviral activity of sulforaphane (SFN), the principal biologically active phytochemical of broccoli extract. SFN inhibited in vitro replication of six strains of SARS-CoV-2, including Delta and Omicron variants. Prophylactic administration of SFN to K18-hACE2 mice prior to intranasal SARS-CoV-2 infection significantly decreased the viral load in the lungs and upper respiratory tract and reduced lung injury and pulmonary pathology. SFN treatment diminished immune cell activation in the lungs, including significantly lower recruitment of myeloid cells and a reduction in T cell activation and cytokine production as well [22]. These results combined with our previous findings on the anti-SARS-CoV-2 efficacy of the micronutrient complexes [23] illustrate potential benefits of applying specific combinations of individual components in addressing complex metabolic pathologies with each particular ingredient having distinct metabolic specificity, and their interactions can expand cellular efficacy through synergistic and complementary effects. In addition, application of natural compounds as multi-nutrient combinations also allows achieving desired results with lower doses of individual compounds compared to their use as separate ingredients. A lack of, or negligent side effects of, natural compounds are important for their potential application in preventive and therapeutic aspects of infection. Considering the urgency in finding an effective prevention, but also the side effects of COVID-19 treatments, our results call for clinical confirmation of the benefits of this micronutrient combination in both preventive and therapeutic aspects of SARS-CoV-2 infection. The advantage of using this combination of micronutrients compared to a single nutrient or a drug therapy is that it can affect several mechanisms of SARS-CoV-2 at once, it displays similar efficacy against various forms of this virus, and since it is based on natural compounds it is potentially free of unwanted side effects.

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