Inhibitory effects of specific combination of natural compounds against SARS-CoV-2 and its Alpha, Beta, Gamma, Delta, Kappa, and Mu variants

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ABSTRACT

Despite vaccine availability, the global spread of COVID-19 continues, largely facilitated by emerging SARS-CoV-2 mutations. Our earlier research documented that a specific combination of plant-derived compounds can inhibit SARS-CoV-2 binding to its ACE2 receptor and controlling key cellular mechanisms of viral infectivity. In this study, we evaluated the efficacy of a defined mixture of plant extracts and micronutrients against original SARS-CoV-2 and its Alpha, Beta, Gamma, Delta, Kappa, and Mu variants. The composition containing vitamin C, N-acetylcysteine, resveratrol, theaflavin, curcumin, quercetin, naringenin, baicalin, and broccoli extract demonstrated a highest efficacy by inhibiting the receptor-binding domain (RBD) binding of SARS-CoV-2 to its cellular ACE2 receptor by 90%. *In vitro* exposure of test pseudo-typed variants to this formula for 1 h before or simultaneously administrated to human pulmonary cells resulted in up to 60% inhibition in their cellular entry. Additionally, this composition significantly inhibited other cellular mechanisms of viral infectivity, including the activity of viral RdRp, furin, and cathepsin L. These findings demonstrate the efficacy of natural compounds against SARS-CoV-2 including its mutated forms through pleiotropic mechanisms. Our results imply that simultaneous inhibition of multiple mechanisms of viral infection of host cells could be an effective strategy to prevent SARS-CoV-2 infection.

INTRODUCTION

Despite availability of vaccines and drastic public health measures, the spread of SARS-CoV-2 infection continues [1]. According to the WHO, as of August 31, 2021, there have been almost 217 million cases worldwide, resulting in more than 4.51 million deaths [2].

Within one year of the pandemic, several variants of SARS-CoV-2 virus have emerged including: United Kingdom B.1.1.7 (Alpha) variant, South Africa B.1.315 (Beta), Brazil P.1 (Gamma), and mutated forms from India known as lineage B.1.617.1 (Kappa) and B.1.617.2 (Delta) [3]. All of these variants spread to other countries, with each mutation potentially giving rise to further variants such as originated in Japan R.1 variant having a mutation E484K located in the spike protein giving "increased resistance" to antibodies generated by the current vaccines [4], as well as a D614G mutation giving increased infectiousness and present in all other variants that have surpassed the Alpha strain [5]. Clinical reports also show that the British variant can infect patients, who have received the vaccine developed against the original SARS-CoV-2, thereby challenging any claim of its universal efficacy [6].

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There are recent indications that the newly emerged Mu variant and R.1 variant may escape antibodies generated by the application of vaccines against original SARS-CoV-2 [7]. Thus, it is foreseeable that the ultimate control of the ongoing COVID-19 pandemic caused by the rapidly emerging mutations of SARS-CoV-2 will be compromised by the need to develop new vaccines potentially for every new variant, and by the related scientific, economic, and social consequences of such a strategy.

The cellular entry mechanism of SARS-CoV-2 has been extensively studied and found to be mediated by a spike protein anchored on the surface of the virus. The spike protein is a trimer, with three receptor-binding S1 heads sitting on top of a trimeric membrane fusion S2 stalk [8]. The spike S1 protein on SARS-CoV-2 contains a receptor-binding domain (RBD) that binds to a target cell using the human ACE2 (hACE2) receptor present on the surface of many types of cells and is proteolytically activated by human proteases. Entry into host cells is an important determinant of viral infectivity and pathogenesis [9–11].

Cellular infection by the SARS-CoV-2 is facilitated by several host enzymatic proteins, including transmembrane protease serine 2 (TMPRSS2), furin, cathepsin L, as well as RNA-dependent RNA polymerase (RdRp) catalyzing viral RNA multiplication [12]. The RNA-dependent RNA polymerase (RdRP) is a conservative in the structure viral enzyme with no host cell homologs and as such it has been of interest in developing selective inhibitors as a potentially safer alternative for treating COVID-19 and not affecting human host cells proteins directly [13].

Application of natural compounds with their wide pleiotropic effects provides an opportunity of controlling several mechanisms associated with SARS-CoV-2 infection with a high margin of safety. Since the emergence of COVID-19, several studies have shown the efficacy of such compounds as inhibitors [14–17]. Our earlier studies also identified the efficacy of plant-derived compounds on several cellular mechanisms of SARS-CoV-2 infectivity [18–20]. In this study, we evaluated the efficacy of a specific combination of micronutrients and plant extracts against newly emerged mutated forms of SARS-CoV-2 and the activity of key enzymes involved in viral processing in human alveolar cells. This approach aims at simultaneous inhibition of multiple cellular mechanisms involved in SARS-CoV-2 infectivity, irrespective of mutation and/or subtype, thereby opening new avenues towards the global control of the pandemic.

MATERIALS AND METHODS

Cell lines, virial particles, and antibodies

A549 and VeroE6 cells were obtained from American Type Culture Collection (Manassas, VA, USA). The hACE2/A549 cells (i.e., A549 cells stably overexpressing human ACE2 receptor) and eGFP-luciferase-SARS-CoV-2 spike protein encapsulated pseudo-typed particles were purchased from GenScript (Piscataway, NJ, USA). Pseudo-typed ΔG-luciferase (G*ΔG-luciferase) rVSV was obtained from Kerafast (Boston, MA, USA). Alpha, Beta, Gamma, Delta, Kappa, Mu, and bald eGFP-luciferase-SARS-CoV-2 pseudo-typed particles were from BPS Bioscience (San Diego, CA, USA). All other compounds were from Sigma (St. Louis, MO, USA) except for broccoli extract that was from Bulk Supplements (Henderson, NV, USA).

Plant-derived compositions

The combinations of natural compounds tested in this study are presented in Table 1. Stock solutions of these combinations were prepared in dimethylsulfoxide (DMSO) at $50 \, mg \, mL^{-1}$ and kept at $-20 \, °C$ until analysis. For the experiments, the stock solution was diluted with $1 \times$ phosphate buffered saline ($1 \times$ PBS) or a buffer recommended by the manufacturer of the utilized kit (enzyme activity assays) or corresponding cell culture medium (cell-based experiments) to final concentrations indicated in the Figures.

Binding of SARS-CoV-2 pseudo-typed virions to hACE2 receptor

The experiment followed our previously published methodology [18, 19]. Briefly, original eGFP-luciferase-SARS-CoV-2 spike protein encapsulated pseudo-virion or its variants were incubated at $37 \, °C$ with $0–100 \, µg \, mL^{-1}$ of MixV for either 1 h before it was added into a monolayer of hACE2/A549 cells or added simultaneously to hACE2/A549 cells. Cells were incubated for an additional 48 h at $37 \, °C$. After the 48-h incubation period, the transduction efficiency was quantified by recording the luciferase activity, utilizing a luciferase assay system (Promega, Madison, WI, USA) and a spectrofluorometer (Tecan Group Ltd., Männedorf, Switzerland). The positive control was bald eGFP-luciferase-SARS-CoV-2 pseudo-typed particles, and the negative control was ΔG-luciferase rVSV pseudo-typed particles. Data are presented as a % of control without Mix-V addition (mean ± standard deviation (SD), $n = 5$).

In vitro RdRp activity assay

In vitro RdRp activity was evaluated using a SARS-CoV-2 RNA Polymerase Assay Kit (ProFoldin, Hudson, MA, USA)

Table 1. Combinations of plant extracts and micronutrients tested in the study

Substance	MixA	MixB	MixC	MixV	MixE
Green tea extract	+	+	+		
Resveratrol	+	+	+	+	+
Broccoli extract	+	+	+	+	+
Curcumin	+	+	+	+	+
Quercetin	+	+	+	+	+
Naringenin	+	+	+	+	+
Baicalin	+	+	+	+	+
Theaflavin	+			+	+
Vitamin C	+	+	+	+	+
N-acetylcysteine		+		+	
Fucoidan			+		+

according to the manufacturer's protocol. Briefly, 0.5 μL of 50 × recombinant RdRp or 20 μL of cell lysate [to transduce cells with eGFP-luciferase-SARS-CoV-2 RdRp lentivirus (GenScript, Piscataway, NJ, USA), VeroE6 cells were seeded into a 6-well plate in the presence of complete growth medium and treated with 8 μL mL^{-1} polybrene (Sigma, St. Louis, MO, USA) for 30 min, followed by the addition of eGFP-luciferase-SARS-CoV-2 RdRp lentivirus at multiplicity of infection (MOI = 5) [our previous preliminary results showed an almost 100% transduction rate can be achieved with this MOI], and spin-inoculation at 1,000×g for 1.5 h. After 24 h at 37 °C incubation, cells were fed with fresh complete growth medium. After 48 h post-inoculation, cells were detached with 1 mM EDTA, washed three times with 1 × PBS supplemented with 3% fetal bovine serum (FBS)] was incubated with 2.5 μL of 50 × buffer, 20 μL of water (only when recombinant RdRp enzyme was used), and 1.0 μL of MixV 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 × NTPs and 0.5 μL of 50 × 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 × fluorescence dye, and the fluorescence signal was recorded in 10 min at extension/emission = 488/535 nm using a fluorescence spectrometer (Tecan, Group Ltd., Männedorf, Switzerland). Positive control contained 100 μg mL^{-1} remdesivir. Results are expressed as a % of control without MixV addition (mean ± SD, $n = 5$).

Furin activity assay

Effects of MixV on furin enzymatic activity were evaluated using a SensoLyte Rh110 Furin Activity Assay Kit (AnaSpec, Fremont, CA, USA) according to the manufacturer's protocol. Briefly, MixV at 5 and 10 μg mL^{-1} concentrations was mixed with furin recombinant protein for 15 min, followed by the addition of fluorogenic Rh110 furin substrate. The samples were incubated for 1 h at 22 °C and the fluorescence was recorded at extension/emission = 490/520 nm with a fluorescence spectrometer (Perseptive Biosystems Cytofluor 4000, Framingham, MA, USA). Positive control as a peptidyl chloromethylketone (Decanoyl-Arg-Val-Lys-Arg-CMK) contained 0.1 μM. Data are presented as a % of control without MixV addition (mean ± SD, $n = 5$).

Cathepsin L activity assay

Effect of MixV on the activity of recombinant cathepsin L was evaluated using a Cathepsin L Activity Screening Assay Kit (BPS Bioscience, San Diego, CA, USA), according to the manufacturer's protocol. Briefly, MixV at 5 and 10 μg mL^{-1} concentrations was added to cathepsin L (0.2 mU/μL) for 15 min at 22 °C prior to fluorogenic substrate (Ac-FR-AFC) (10 μM) addition and incubation for further 60 min at RT. Positive control contained only cathepsin L, and negative control contained cathepsin L and cathepsin L inhibitor E-64 (25 μM). The fluorescence was recorded at extension/emission = 360/480 nm with a fluorescence spectrometer (Tecan Group Ltd., Männedorf, Switzerland). Data are

presented as a percentage of control without MixV addition (mean ± SD, $n = 5$).

Viability

Cell viability assay was performed using MTT substrate. Briefly, 40 × 10^3 A549 cells per well were treated with different concentrations of MixV alone or in combination with vitamin D and zinc for up to 48 h. Subsequently, the wells were washed with 1 × PBS and complete growth medium supplemented with 5 mg mL^{-1} MTT was added, followed by incubation for 4 h at 37 °C. Next, the culture medium was aspirated and 100 μL of methanol was added. The absorbance was assessed at 570 nm with a fluorescence spectrometer (Molecular Devices, San Jose, CA, USA). Data are presented as a % of control without MixV 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.

RESULTS

Selection of the most effective composition of natural compounds based on its inhibition of viral RBD binding to hACE2 receptor

The results in Fig. 1 show the efficacy of various combinations of plant extracts and micronutrients on inhibition of SARS-CoV-2 RBD binding to epithelial pulmonary cells over-expressing human ACE2 receptor. All five compositions displayed inhibitory effects on RBD binding at the level of 50% and higher. The highest inhibition, i.e., by 90% compared to control, was obtained with MixV applied at 10 μg mL^{-1} concentration (Fig. 1A). The addition of vitamin D or zinc to MixV did not further enhance the inhibition of RBD binding to human ACE2 receptor as shown in Fig. 1B.

Effect of MixV on cellular attachment and entry of SARS-CoV-2 and its pseudo-typed variants

The effect of different pattern of applications of MixV on the attachment and cellular entry of eGFP-luc-spike-enveloped pseudo-typed variants was tested using A549/hACE2 cells. The results presented in Fig. 2A show that the exposure of pseudo-virions to MixV before their infection of human lung cells was effective in decreasing cellular entry of all tested SARS-CoV-2 variants. As such, the MixV applied at 10 μg mL^{-1} to original SARS-CoV-2 strain and its Alpha,
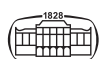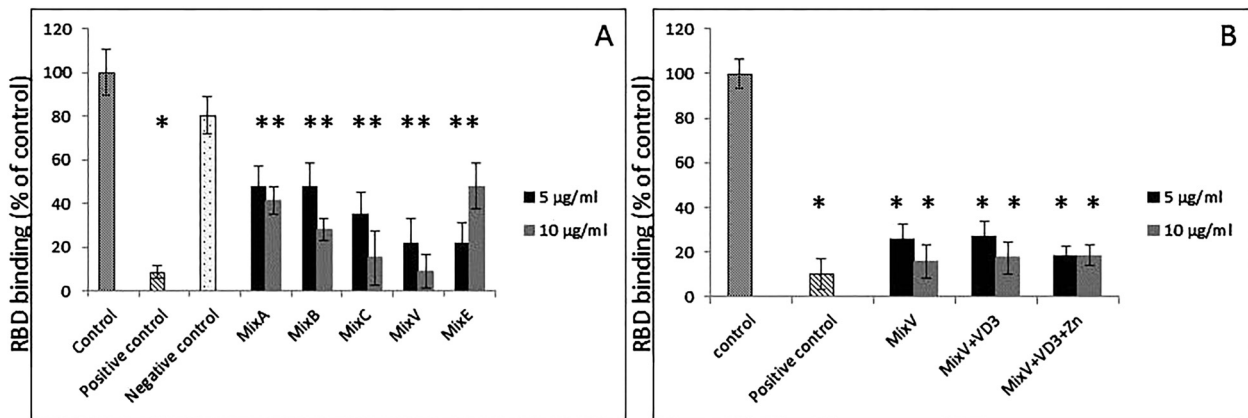
Fig. 1. *Effect of test compositions on SARS-CoV-2 RBD-spike protein attachment to lung epithelial cells.* Inhibitory effect of Mixes A, B, C, V, and E on viral SARS-CoV-2 RBD binding to human ACE2 receptor (A). Inhibitory effect of MixV in the presence of vitamin D and zinc on viral SARS-CoV-2 RBD binding to human ACE2 receptor (B); control – 0.25% DMSO, positive and negative controls was provided by the manufacturer of the kit; data are presented as % of control $\pm$ SD; * $P \leq 0.001$; VD3 – 25-hydroxy Vitamin D_3 at 25 μg mL^{-1}, Zn – zinc aspartate at 2.5 μg mL^{-1}
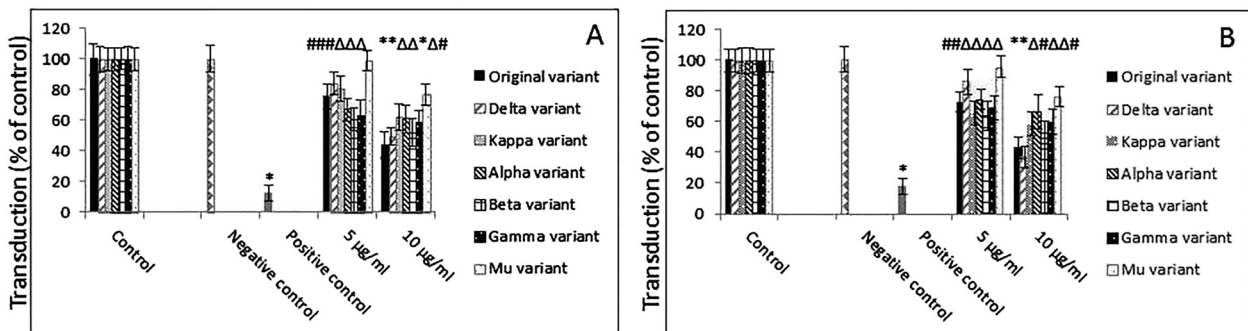
Fig. 2. *Effect of MixV on SARS-CoV-2 eGFP-luciferase pseudo-typed variants entry.* hACE2/A549 cells were inoculated with six different variants of SARS-CoV-2 pseudo-virions encapsulated with spike proteins and treated with MixV at designated concentrations either as a pre-treatment (A) or as a simultaneous exposure (B). Control – 0.05% DMSO, negative control – pseudo-typed ΔG-luciferase (G*ΔG-luciferase) rVSV, positive control – bald SARS-CoV-2 eGFP-luciferase pseudo-virions; # $P \leq 0.05$, Δ $P \leq 0.01$, * $P \leq 0.001$

Beta, Gamma, Delta, Kappa, and Mu variants for 1 h before their adding to A549/hACE2 cells, resulted in decreased viral entry by 39%, 48%, and 42%, 57%, 51%, 39%, and 24%, respectively. Figure 2B shows the effects of MixV on the test variants cellular entry inhibition when it was applied simultaneously with pseudo-typed virions and A549/hACE2 cells. In the presence of MixV at 10 μg mL^{-1} concentration, the cellular entry of Alpha virions was inhibited by 38%, Beta by 48% and Gamma by 41%. The inhibitory effect of the MixV on original SARS-CoV-2 virions entry was 57% and Delta, Kappa and Mu variants 64%, 42%, and 24%, respectively. The results presented on Fig. 3A show that MixV applied at 5 and 10 μg mL^{-1} was not affecting viability of A549/hACE2 cells and the administration of vitamin D3 alone or with zinc did not have additional impact on their survival (Fig. 3B).

Effect of MixV on viral RdRp polymerase

In our study we also tested whether MixV acts beyond the binding steps of the SARS-CoV-2 infection process, by examining its effect on other important cellular mechanisms in viral infection, such as the activity of RdRp, which is essential for viral replication. As presented in Fig. 4A, enzymatic activity of recombinant SARS-CoV-2 RdRp was significantly inhibited by 53% in the presence of MixV at 10 μg mL^{-1}. Neither Vitamin D3 nor zinc enhanced this inhibitory effect. A similar inhibitory effect of MixV on RdRp activity was also observed when using the lysate of cells overexpressing RdRp protein (Fig. 4B).

Effect of MixV on host cellular proteases

It has been documented that various specific host cell proteases, including furin and cathepsin L, are required to facilitate SARS-CoV-2 cellular entry by enzymatic "priming" spike proteins and viral egress. In our study, we applied cell-free assays to study direct effects of MixV on activity of these enzymes. As presented in Fig. 5A, we observed inhibition of the activity of furin by 12% and 34% with MixV applied at 5 and 10 μg mL^{-1}, respectively. This inhibitory effect of MixV was further enhanced in the presence of vitamin D resulting
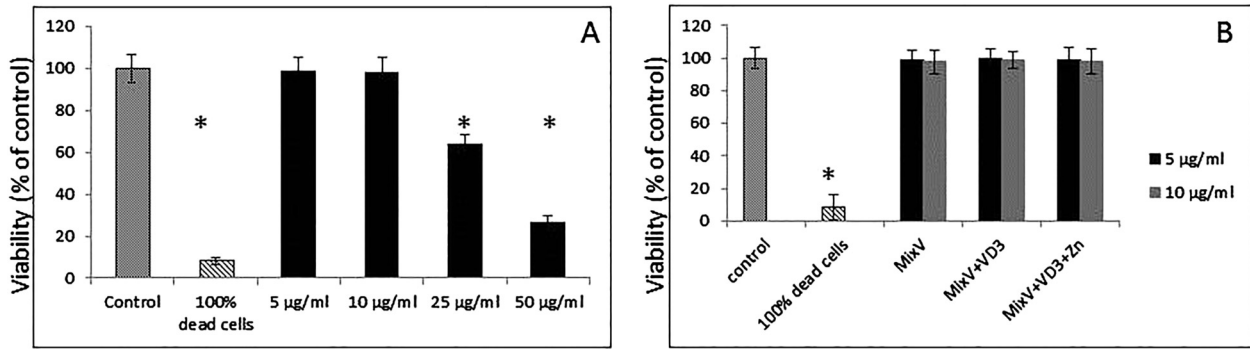
Fig. 3. *Viability of human lung epithelial cells.* Effects of MixV on survival of A549/hACE2 cells (A). Effects of MixV its combination with vitamin D3 and zinc on survival of A549/hACE2 cells (B). Viability of A549/hACE2 cells was assessed using MTT method as described in Experimental Procedures section; controls – 0.05% DMSO; * $P \le 0.001$ VD3 –25-hydroxy Vitamin D_3 at $25 \, \mu g \, mL^{-1}$, Zn – zinc aspartate at $2.5 \, \mu g \, mL^{-1}$
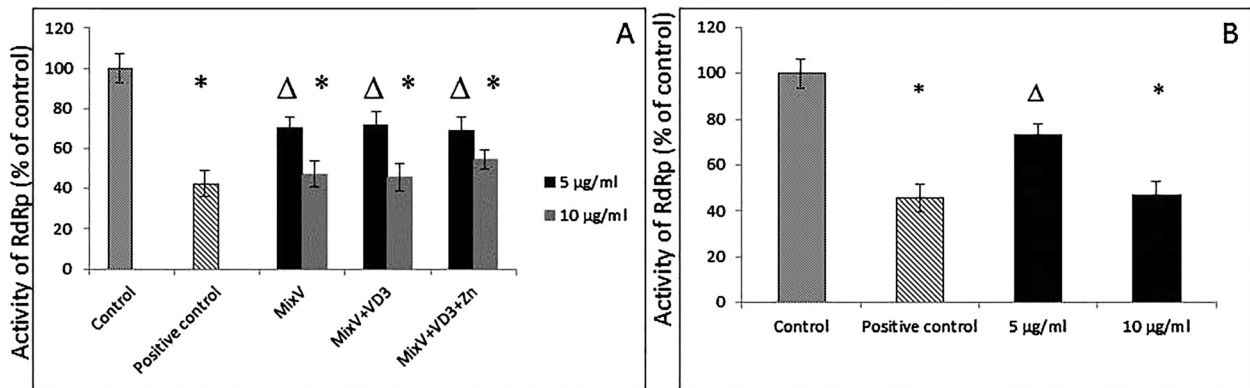
Fig. 4. *Effect of MixV on viral RdRp.* Effects of MixV alone (A) and in combination with vitamin D3 and zinc (B) on activity of SARS-CoV-2 RdRp. Purified recombinant RdRp enzyme in VeroE6 cells was incubated with MixV at designated concentrations for 15 min at RT. After application of reaction mix composed 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, positive control – $100 \, \mu g \, mL^{-1}$ remdesivir; $\Delta \, P \le 0.01$, * $P \le 0.001$; VD3 – 25-hydroxy Vitamin D3 at $25 \, \mu g \, mL^{-1}$, Zn – zinc aspartate at $2.5 \, \mu g \, mL^{-1}$
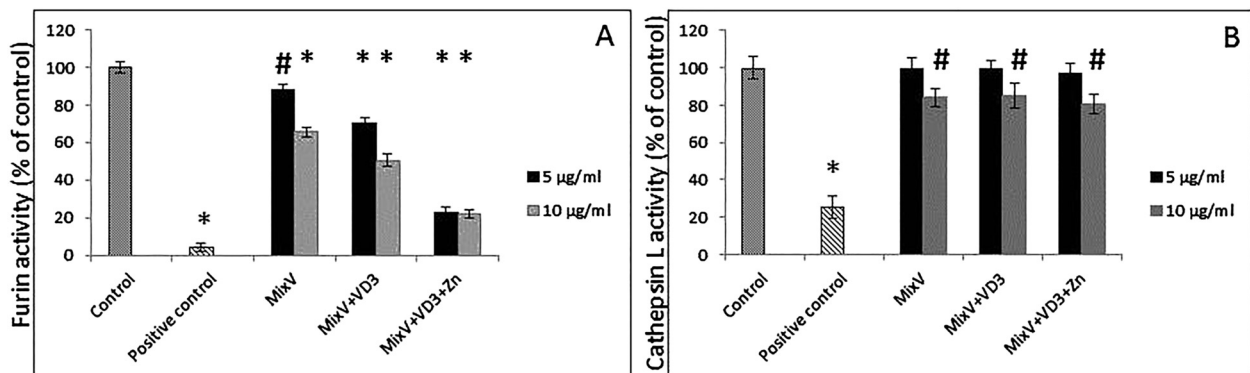

Fig. 5. *Effect of MixV on cellular proteases.* Effect of MixV on furin activity (A). Furin as a recombinant protein was incubated with MixV alone or in combination with vitamin D and zinc at indicated concentrations for 15 min followed by addition of fluorogenic Rh110 furin substrate, and incubation for 1h at 22 °C. Effect of MixV on cathepsin L activity (B and C). Cathepsin L as a recombinant protein was incubated with MixV alone or in combination with vitamin D and zinc at indicated concentrations for 1 h at 22 °C followed by the application of $10 \, \mu M$ of fluorogenic substrate for 30 min. Control – 0.05% DMSO, positive control – $25 \, \mu g \, mL^{-1}$ E-64; # $P \le 0.05$, * $P \le 0.001$, VD3 – 25-hydroxy Vitamin D_3 at $25 \, \mu g \, mL^{-1}$, Zn – zinc aspartate at $2.5 \, \mu g \, mL^{-1}$

in 54% inhibition. A combination of MixV with vitamin D and zinc even further decreased furin activity by 78%. As shown in Fig. 5B, the MixV, although mildly, also was effective in decreasing activity of cathepsin L involved in SARS-CoV-2 endosomal egress. In the presence of 10 μg mL^{-1} MixV enzymatic activity of cathepsin L was reduced by 14%, and addition of vitamin D3 did not have a significant effect on cathepsin L inhibition.

DISCUSSION

Our study represents a new comprehensive approach to COVID-19 with its aim at controlling not one but several cellular mechanisms associated with SARS-CoV-2 infectivity at once. Our results show that a defined combination of active plant components and selected micronutrients (MixV) can significantly affect critical steps required for cellular infectivity of the original SARS-CoV-2 strain and its mutated forms Alpha, Beta, Gamma, Delta, Kappa, and Mu, by inhibiting viral attachment to cellular ACE2 receptors, decreasing availability of these receptors on human lung cells, and decreasing activity of viral RdRp necessary for viral replication. In addition, these natural compounds interfered with cellular entry of the virions by decreasing activity of important enzymes such as furin and cathepsin L (see Fig. 6).

These findings further expand on our earlier study using SARS-CoV-2 virion particles, which identified core ingredients in this combination, which were effective in inhibiting viral binding to hACE2 receptor and decreasing expression of ACE2 receptors on human small alveolar epithelial cells [21]. Taking into account immediate availability and high safety profile of these natural compounds, their application as preventive measures, e.g. as an adjunct to vaccines and therapies of COVID, is plausible.

While all tested nutrient combinations (A, B, C, D and E) were effective in blocking RBD binding to ACE2 by about 50%, the MixV demonstrated the highest, 90% inhibition of viral binding. This would indicate that synergistic or at least additive effect could play a role here, not excluding other,

e.g., allotropic, interactions between the compounds of MixV and RBD/ACE2.

Our study investigated inhibitory effects of MixV on cellular internalization of both SARS-CoV-2 virions and its mutated forms using different pattern of exposure of these virions to MixV and human lung cells. As such, we incubated test virions with MixV for 1 h before their exposure to the cells and combined MixV with virions and cells simultaneously. The results indicate that maximum inhibition of cellular transduction by these virions can be obtained when all of these elements interacted at the same time. This would imply that this mixture affects metabolic aspects of cell-virions interactions rather than acts on the virus itself. Although its efficacy in inhibiting viral internalization was not virus specific, MixV was nevertheless most effective in inhibiting the original SARS-CoV-2 and Delta variants compared to other mutated forms.

In addition to the inhibitory effect of MixV on cellular attachment and entry of SARS-CoV-2 virions, we also observed that MixV affected cellular mechanisms involved in endosomal egress and multiplication of the virus by decreasing activity of enzymes involved in viral cellular processing. Among the proteolytic enzymes involved in these processes, the activity of cathepsin L and furin has been shown to be important for pathogenicity of SARS-CoV-2 [22, 23]. It also has been shown that furin action towards the SARS-CoV-1 spike protein is necessary for the fusion of virions with host membranes, without directly affecting viral infectivity [24]. It appears that effective control and treatment of SARS-CoV-2 infection might necessitate simultaneous inhibition of several proteases to effectively obstruct these pathological conversions.

Here we have shown that, in addition to impairing viral binding to hACE2 overexpressing lung epithelial cells, the MixV decreased activity of membrane protease furin and endosomal cathepsin L. The reduction of the activity of these enzymes was observed at non-toxic MixV concentrations. This effect is significant, as the lack of the additional furin cleavage site on the SARS-CoV-1 spike protein has a substantial influence on its infectivity [25].
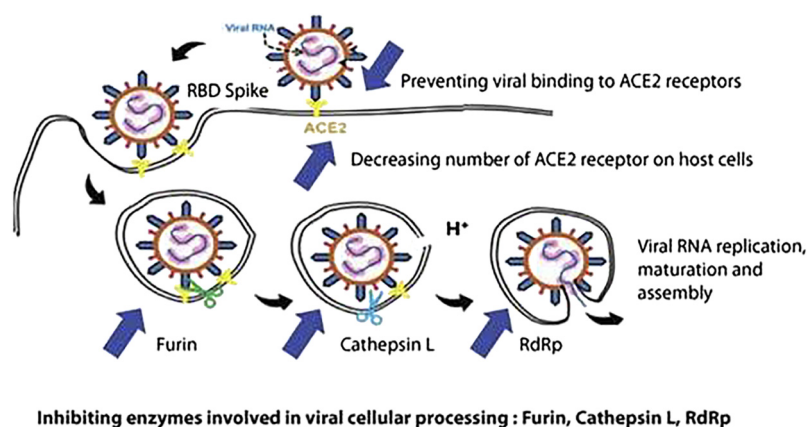

Fig. 6. Schematic representation of the effects of MixV on key cellular processes associated with SARS-CoV-2 infectivity

In addition to SARS-CoV-2 infection, the potential signal link between spike protein, furin, and ACE2 has been implied in the occurrence of adverse cardiovascular events [26]. Since these enzymes are also involved in various biochemical pathways, a lack of complete inhibition at the concentrations of MixV used in the study may be beneficial. We observed that vitamin D3 and zinc had additional effect on lowering enzymatic activity of furin, but not cathepsin L. Finally, we also recorded a substantial inhibition of activity of viral RdRp at these concentrations, with its consequences at reduced replication rate of the virus.

Based on this study and our earlier findings [18, 19, 27], this combination of plant-derived compounds and micronutrients may constitute a new anti-SARS-CoV-2 strategy by simultaneously affecting multiple aspects of viral life cycle, including viral entry and replication. This strategy was also implemented in our earlier studies, including those of human influenza H1N1, bird flu H1N5, and others, which were based on selecting natural components that simultaneously affect key pathology mechanisms across a wide spectrum of infective agents [28–31].

Our study implies that a simultaneous control of key steps of the SARS-CoV-2 infectivity by a definite combination of plant-derived and biologically active compounds can be effective in addressing complex pathologies, such as involved in this viral infection. Taken together, with the recently established clinical evidence that vitamin C infusions can successfully combat COVID-19 even at advanced stages [32], phyto-compounds through their pleiotropic biological effects could represent a new approach against SARS-CoV-2 and its mutants.

ACKNOWLEDGMENT

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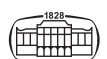
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