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Epigallocatechin Gallate and Isoquercetin Synergize With Remdesivir to Reduce SARS-CoV-2 Replication *In Vitro*

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The ongoing pandemic of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) urgently needs effective antivirals. After over 2 years since the beginning of the pandemic, only a few FDA approved therapeutic options are available to treat the population. Combination therapies have become a standard for the treatment of other infectious diseases such as HIV and hepatitis C due to their improved efficacy compared to monotherapy, reduced toxicity, the ability to prevent the development of resistant viral strains and their potential to treat co-infection. The interest in identifying molecules displaying bioactivity against SARS-CoV-2 has led to extensive search for promising molecules from the natural pharmacopoeia and polyphenols have been shown to display antiviral activity against a number of viruses including SARS-CoV-2. Here we evaluated the *in vitro* efficacy of two polyphenols, Epigallocatechin gallate (EGCG) and Isoquercetin, in combination with Remdesivir, the first-approved drug for the treatment of severe COVID-19. We confirmed the inhibitory effects of EGCG and isoquercetin against SARS-CoV-2 and demonstrated their strong antiviral synergistic effects with Remdesivir *in vitro*. These combinational therapies represent an interesting avenue for the treatment of COVID-19 and grant further studies.

Keywords: SARS-CoV-2, polyphenols, remdesivir, combinational therapy, synergistic effect, *in vitro*

INTRODUCTION

The Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) outbreak is causing an unprecedented global pandemic that has led to an unparalleled worldwide public health emergency. As of June 2022, the scale of the coronavirus disease 2019 (COVID-19) pandemic, with over 544 million cases and 6.3 million deaths globally over a 2-year period (1) exceeds the 1968 influenza A/H3N2 pandemic, which reported ~1 million deaths worldwide. Patients with COVID-19 can develop pneumonia (2), severe symptoms of acute respiratory distress (ARDS), and multiple organ failure (3). This RNA virus is related to SARS-CoV-1, which emerged in 2002-03 in China, but has distinct features provoking specific morbid symptoms. Infected persons display strong respiratory

syndrome including fever, cough, shortness of breath and dyspnea. Severe symptoms will lead to pneumonia and are associated with a major inflammatory crisis in the lungs which leads to improper alveolar pulmonary function and poor gas exchange and blood oxygenation. The disease can also lead to kidney failure and death. Increasing evidence shows that immune patterns are closely associated with the disease progression of patients infected with SARS-CoV-2, where the altered responses are due to both replication in different organs as well as the exacerbation of the inflammatory reactions. This outcome is even more important in high-risk individuals and elderly people (4).

While there are currently multiple effective vaccines, there is still a considerable amount of the global population which has not yet received any dose of COVID-19 vaccine due to vaccine hesitancy and public mistrust, no equitable access to vaccines in many countries, or not yet eligible for vaccination. In addition, immunocompromised individuals can have reduced responses to vaccines, due to their weak immune system. Therefore, the combination of these events is promoting the emergence of viral variants that are increasingly transmissible and are evolving to escape human immunity, as it is the case with omicron variant and its different sub-lineages (5). There is, thus, an immediate unmet need for antivirals that can be rapidly used to treat COVID-19 patients in the unvaccinated, the immunocompromised, and those infected with virus variants that escape human immunity.

COVID-19 antiviral therapy plays a pivotal role in limiting the burden of diseases, preventing infection and minimizing household or community transmission, reducing long-term sequelae of the disease, as well as reducing hospitalization, thus, having an important impact on public health. Very few therapeutic options are available for the treatment of COVID-19 patients, including monoclonal antibodies, which are in general very expensive, and recently, two antivirals, Paxlovid and Molnupiravir, have been authorized in the US. However, these antivirals have significant caveats, including low supply and use only among patients at high risk of severe illness and death.

Remdesivir (GS-5734) is an inhibitor of the viral RNA-dependent RNA polymerase with *in vitro* inhibitory activity against SARS-CoV-2 and other coronaviruses such as SARS-CoV-1 and the Middle East respiratory syndrome (MERS-CoV) (6, 7). Remdesivir has been granted emergency or conditional authorization in the U.S., Europe, Australia, Singapore, Japan and Canada for people with severe symptoms (8) as a promising treatment option to reduce hospitalization by an average of 4 days (9). The current available Remdesivir requires intravenous administration, however, an orally bioavailable nucleoside prodrug (GS-621763) has been designed for optimal delivery of the parent nucleoside (GS-441524) into the systemic circulation and is being evaluated in *in vitro* and *in vivo* models (10).

In the context of the actual pandemic, it is urgent to revisit the arsenal of already developed synthetic drugs and natural molecules from the traditional pharmacopoeia with potential action against the virus. The latter category appears promising to fight viruses and especially SARS-CoV-2. In particular, polyphenols have been shown to display antiviral activity against a number of viruses such as influenza (11–14), Zika (15), Ebola (16), Dengue (17, 18), hepatitis (19), HIV (20, 21) and many others (22–24). As a matter of fact, flavonoids like epigallocatechin gallate (EGCG), present in green tea, display strong antiviral activity against many human viruses including HIV type I, Human T-cell lymphotrophic virus type I, Hepatitis B and C viruses, Herpes types 1 and 2, Epstein-Barr virus, Human Papilloma virus, Influenza virus, rotavirus, and several enteroviruses (25–33). The flavanone hesperidin, present in citruses, has also been shown to inhibit the replication of influenza virus type A (H1N1) (34, 35), and rotaviruses (29), yet, not as efficiently as EGCG. Oligognol™, a catechin oligomer (proanthocyanidin) extract found in the litchi exocarp, blocks the fixation of influenza A viruses to its receptor on Madin-Darby Canine Kidney cells and suppresses the nuclear transport of viral ribonucleoprotein (RNP) (36). Finally, quercetin glycosides (i.e. quercetin-3-β-O-d-glucoside or Isoquercetin, quercetin-4-o-β-glucoside, rutin, quercetin-sophoroside) present in apples, onions, and many Brassicas have been shown to prevent Ebola infection in Vero cells and in murine models (16). Quercetin, also prevents the replication of Zika virus (37), Dengue (38) and influenza A/H1N1 viruses (39).

The renewed interest in identifying molecules displaying bioactivity against SARS-CoV-2 has led to extensive search for promising molecules from the natural pharmacopoeia. Using computational molecular docking and in silico binding affinity, candidate molecules targeting SARS-CoV-2 proteins (PLpro, 3CLpro, RNA-dependent RNA polymerase (RdRp), helicase, Spike) have been tested for their potential use for inhibiting replication of this virus (40–44). Using this strategy, many molecules belonging to the flavonoid family, like EGCG, quercetin, hesperidin, chrysin and baicalain, all flavonoids, have shown potential (45), thus warranting further *in vitro* testing.

Based on this preliminary information, we investigated the *in vitro* antiviral activity of selected polyphenols against SARS-CoV-2 and we provide evidence for their synergistic effect when used concomitantly with Remdesivir, the first-approved drug for the treatment of severe COVID-19 (8).

MATERIALS AND METHODS

Cells and Virus

To assess the ability of polyphenols to inhibit SARS-CoV-2 replication *in vitro*, we used Vero E6 cells (ATCC® CRL-1586™) maintained at 37°C, 5% of CO_2 in Minimum Essential Medium (Gibco, Paisley, UK) supplemented with HEPES (Wisent, Québec, CA) and 10% of fetal bovine serum (Cytiva, Utah, USA).

The wild-type virus SARS-CoV-2/Quebec City/21697/2020 used for the experiments was recovered from a patient in April 2020 and passaged twice in Vero E6 cells to produce a stock of the virus. All manipulations using SARS-CoV-2 were carried out at the Containment Level 3 (CL3) laboratory at the CHU de Québec-Université Laval Research Center.

Antiviral Compounds

EGCG and Isoquercetin (Quercetin-3-O-glucopyranoside) were obtained from Sigma-Aldrich (St Louis. MO, USA) and 10 mM stock solution were prepared in DMSO. Remdesivir was purchased from MedChem Express (Princeton, NJ, USA) and 100 mM stock solution was prepared in DMSO.

In Vitro Antiviral Analyses

Determination of Antiviral Effective Concentration Inhibiting Virus Replication by 50% (EC_{50}) Using Monotherapy

Vero E6 cells (30000 cells per well) were cultured in 96-well plates. One day later, cells were infected with 200 Median Tissue Culture Infectious Dose ($TCID_{50}$) of SARS-CoV-2 per well. After 1 hour of incubation at 37°C in a 5% of CO_2 atmosphere, antivirals in two-fold serial dilutions were added with final concentrations ranging from 15.6 μM to 125 μM for EGCG, 7.8 μM to 62.5 μM for Isoquercetin and 1.25 μM to 20 μM for Remdesivir. Three days later, cell viability was evaluated using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sul-fophenyl)-2H-tetrazolium (MTS) (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, Nepean, ON, Canada) as previously described (46, 47). Ten microliters per well of MTS was added and absorbance was measured 3 hours later at 490 nm using a Synergy HTX Multi-Mode 96-well plate reader (Agilent). The percentage of cell survival was calculated and the EC_{50} values were determined (48).

Evaluation of Antiviral Combinations

We also combined Remdesivir with the two polyphenols (Remdesivir/EGCG and Remdesivir/Isoquercetin) to evaluate antiviral activity. The EC_{50} of combined polyphenols and antiviral were evaluated using the same principle as for single component. Serial dilutions of the two combinations, with concentrations surrounding the EC_{50} of each molecule tested separately were cross-mixed, resulting in 20 different pairwise comparisons. Cell culture infection and viability were processed as for monotherapy. The EC_{50} of one molecule (considered as the reference molecule) combined with a constant concentration of a second molecule was calculated. Experiments were done twice in quadruplicate. Dose-response curves were performed with Graph-Pad Prism 8.

To evaluate the synergistic effect of antiviral compounds, we used the SynergyFinder software version 2.0 (https://synergyfinder.fimm.fi/synergy/20210225212818919177/). We obtained a dose-response percent matrix inhibition of single and combined compounds (Remdesivir/EGCG and Remdesivir/Isoquercetin) and a 3D interaction surface response between these compounds, and calculated the synergy score for the combination using ZIP (Zero Interaction Potency) reference model as described previously (49). Red areas in the surface response and a synergy score larger than 10 correspond to a synergistic interaction between compounds (https://synergyfinder.fimm.fi/synergy/synfin_docs/#datanal).

Cytotoxicity Assays

Polyphenols and Remdesivir cytotoxic effects were evaluated using the same protocol as for antiviral combinations except for the addition of the virus. Cell viability was calculated and normalized with untreated control, which was defined as 100%.

RESULTS

Inhibition Effects of Remdesivir, EGCG and Isoquercetin Against SARS-CoV-2 Infection

We first determined the 50% effective concentration (EC_{50}) of each compound used in monotherapy. As shown in **Table 1**, EGCG and Isoquercetin were effective to inhibit SARS-CoV-2 replication by 50% at 85.65 ± 6.45 μM and 23.99 ± 1.07 μM, respectively. Isoquercetin has already shown its effectiveness against Influenza and Ebola virus (16, 50) and has been proposed for SARS-CoV-2 treatment (51, 52). Although the EC_{50} of EGCG was relatively high using this assay, it was previously described as effective to block infection of ancestral SARS-CoV-2 and UK-B.1.1.7, SA-B.1.351 and CA-B.1.429 variants, *in vitro* (53). Remdesivir, the first drug approved for severe COVID-19 (8), has been tested in parallel with polyphenols. The EC_{50} of Remdesivir was 9.18 ± 4.88 μM. Another study using Vero cells found a similar EC_{50} value at 8.24 μM (54). These findings confirm the capacity of EGCG, Isoquercetin, and Remdesivir to inhibit SARS-CoV-2 replication.

Synergistic Inhibitory Effect of Remdesivir with EGCG or Isoquercetin Against SARS-CoV-2 Infection

We investigated the antiviral effects of the combination of Remdesivir with EGCG or Isoquercetin by mixing different concentrations of each compound, and calculated the EC_{50} of the reference compound combined with a constant concentration of a second one. The combination of remdesivir and EGCG or Isoquercetin exhibited highly potent synergism as compared with their individual EC_{50}. Considering Remdesivir as reference compound, the addition of 15.65 μM of EGCG or Isoquercetin, reduced the Remdesivir EC_{50} values by at least half (8.18 ± 4.88 μM to 4.42 ± 2.13 and 2.29 ± 0.02, respectively). The EC_{50} was reduced 5 times with 31.5 μM of EGCG (**Table 1**). When EGCG and Isoquercetin concentrations increased, the Remdesivir EC_{50} could not be determined because cell viability was higher than 50% for all concentrations tested. These results indicate that low concentrations of polyphenols, about 5 times of EGCG EC_{50} and 1.5 times of Isoquercetin EC_{50} can potentiate the activity of Remdesivir.

We then used EGCG or Isoquercetin as reference compounds (15.6 to 125 μM and 7.8 to 62.5 μM respectively), and calculated their EC_{50} at a constant concentration of Remdesivir. The addition of much lower concentrations of EGCG-Remdesivir drastically reduced the EC_{50} of EGCG (with 1.25 μM of Remdesivir, 3.1-fold reduction; with 2.5 μM of Remdesivir, 3.4-fold reduction) (**Table 1**). When 5 μM of Remdesivir were added, a concentration below the EC_{50}, we could not determine the EGCG EC_{50} because cell viabilities were higher than 50% for all concentrations tested, indicating a potential effect of EGCG to

TABLE 1 | Antiviral activity of polyphenols (EGCG or Isoquercetin) and Remdesivir alone or in combination against SARS-CoV-2. EC_{50} of one compound alone or combined was determined.

Antiviral	Effective Concentrationthat Inhibits Virus Replication by 50% (μM) SARS-CoV2/Qc/21697/2020
Remdesivir	**9.18 ± 4.88**
Remdesivir (1.25 to 20 μM) + EGCG 15.62 μM	**4.42 ± 2.13**
Remdesivir (1.25 to 20 μM) + EGCG 31.25 μM	**1.79 ± 1.29**
Remdesivir (1.25 to 20 μM) + EGCG 62.5 μM	**<1.25**
Remdesivir (1.25 to 20 μM) + Isoquercetin 15.62 μM	**2.29 ± 0.02**
Remdesivir (1.25 to 20 μM) + Isoquercetin 31.25 μM	**<1.25**
Remdesivir (1.25 to 20 μM) + Isoquercetin 62.5 μM	**<1.25**
EGCG	**85.65 ± 6.45**
EGCG (15.6 to 125 μM) + Remdesivir 1.25 μM	**27.68 ± 14.21**
EGCG (15.6 to 125 μM) + Remdesivir 2.5 μM	**25.02 ± 11.25**
EGCG (15.6 to 125 μM) + Remdesivir 5 μM	**<15.62**
Isoquercetin	**23.99 ± 1.07**
Isoquercetin (7.8 to 62.5 μM) + Remdesivir 1.25 μM	**19.32 ± 5.13**
Isoquercetin (7.8 to 62.5 μM) + Remdesivir 2.5 μM	**17.13 ± 3.19**
Isoquercetin (7.8 to 62.5 μM) + Remdesivir 5 μM	**13.39 ± 1.19**

For compound combinations, two compounds with different concentrations were mixed. EC_{50} for each combination was calculated based on the final concentrations of one serially diluted drug or polyphenol (reference compound) combined with another one maintained at constant concentration. For some combinations, cell survival percentages were greater than 50% for all compound dilutions. Thus, we could not determine the EC_{50} value. In this case, the EC_{50} was considered less than the smallest concentration of the reference compound tested; i.e. < 15.62 μM for combination using EGCG as the reference and <1.25 μM for those using Remdesivir.

improve the antiviral effect of Remdesivir. The EC_{50} of Isoquercetin was also improved with different concentrations of Remdesivir (**Table 1**), but not to a greater extent as with EGCG.

Dose-response curves of compounds tested alone or in combination against SARS-CoV-2 were also evaluated. Serial dilutions of Remdesivir were tested with different constant concentrations of EGCG or Isoquercetin and vice versa. As shown in **Figure 1A**, EGCG with concentration higher than 15.62 μM potentiated the effect of Remdesivir. This finding was observed for Isoquercetin combined with Remdesivir, but only at certain concentrations (1.25 to5 μM of Remdesivir) (**Figure 1B**). Based on serial dilutions of EGCG or Isoquercetin, in combination with different constant concentrations of Remdesivir showed a higher potency for EGCG/Remdesivir than that for Isoquercetin/Remdesivir (**Figures 1C, D**).

Overall, these observations suggested a reciprocal positive effect of EGCG and Isoquercetin with Remdesivir.

To assess the synergistic interaction between Remdesivir and EGCG or Isoquercetin, we used the SynergyFinder web

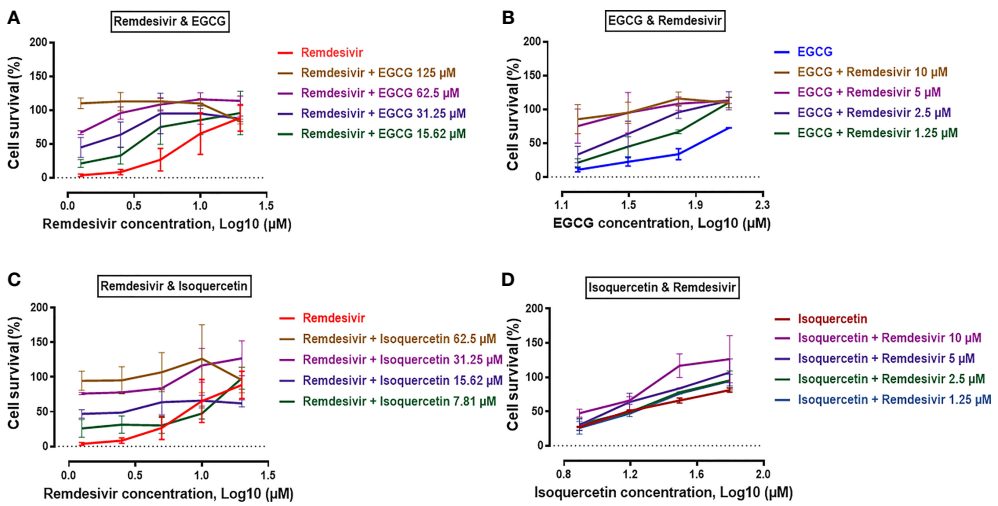

FIGURE 1 | Dose-response curves of antivirals compounds tested alone or in combination against SARS-CoV-2. Serial dilutions of Remdesivir (1.25-20 μM) combined with constant concentrations of EGCG **(A)** or Isoquercetin **(B)**. **(C)** Serial dilutions of EGCG (15.62-125 μM) combined with constant concentrations of Remdesivir. **(D)** Serial dilutions of Isoquercetin (7.81-62.5 μM) combined with constant concentrations of Remdesivir. X axis: Compound concentration in Log10 (μM). Y axis: Percentage of cell survival. Three different experiments were done in quadruplicates each time for both drug combinations.

application (https://synergyfinder.fimm.fi) which generated dose-response matrix, 3D interaction surface response between these compounds and allowed to calculate the synergy score.

For Remdesivir/EGCG combination, the dose-response matrix showed that most of the boxes are red, corresponding to a clear synergistic inhibitory effect of Remdesivir/EGCG against SARS-CoV-2 (**Figure 2A**).

This profile was confirmed by analyzing the 3D interaction surface responses between the polyphenols and the antiviral and indicated a large red area and a synergy score of 30.615 (**Figure 2A**). The dose-response matrix for the combination of Isoquercetin and Remdesivir showed a modest synergistic effect, observed by pallor red boxes and a synergy score of 11.072 (**Figure 2B**).

DISCUSSION

In this study, we aimed to investigate the antiviral effects of the combination of Remdesivir with EGCG or Isoquercetin. We confirmed the inhibitory effects of EGCG and isoquercetin against ancestral SARS-CoV-2 and demonstrated their strong antiviral synergistic effects with Remdesivir *in vitro*. Remdesivir combined with EGCG showed a synergy score of 30.615. This score is high, compared to previous studies testing the Remdesivir combination using Vero cells (55, 56). EGCG interacts with viral membrane proteins and/or cellular proteins and blocks the early stages of infection such as attachment, post-adsorption entry, and genome replication by inhibiting reverse transcriptase, both *in vitro* and *in vivo* (31). Interestingly, EGCG appears to boost the antiviral action of Remdesivir, which means that lower concentration of the latter is needed to provide a similar antiviral activity. This is possibly due to the different mechanism of action of EGCG and Remdesivir. Preliminary data from our group (not published) and others (53, 57) have shown that EGCG inhibit SARS-CoV-2 *in vitro* through their interaction with the virions rather than the cells because pretreatment of virions but not cells with catechin derivatives significantly suppressed the viral infection (53, 57).

Colpitts and Schang (58) have shown that EGCG acts directly on the surface of several virus surface proteins without affecting the fluidity or integrity of the virion envelope by competing with heparan sulfate or sialic acid moieties in cellular glycans preventing virion binding to cells. Additionally, EGCG was also shown to have an inhibitory activity against the SARS-CoV-2 3CL-protease and reduce viral replication in cell culture (59). A multicenter, double-blind, randomized, placebo-controlled clinical trial has been proposed in health care workers directly exposed to clinical care, daily contact, or traffic of individuals with suspected COVID-19 during the epidemic outbreak. The aim of this latter study is to determine the efficacy of Previfenon® (EGCG) to prevent COVID-19, enhance systemic immunity, and decrease the frequency and intensity of selected symptoms when used as pre-exposure chemoprophylaxis to SARS-CoV-2 (https://clinicaltrials.gov/ct2/show/NCT04446065?term=EGCG+%28Previfenon%29&cond=COVID-19&draw=2&rank=1).

The active triphosphate form of Remdesivir acts as a nucleoside analog and inhibits the RdRp of SARS-CoV-2 and other coronaviruses. Remdesivir is incorporated by the RdRp into the growing RNA product and allows the addition of three more nucleotides before RNA synthesis stalls (60). Thus, since targets and mode of actions of each antiviral differ, and are potentiated by one another, we surmise that Remdesivir/EGCG combination could be used as an alternative therapy for immunocompromised patients or for severe cases of COVID-19. EGCG has been shown to inhibit SARS-CoV 3CLproteases with a IC_{50} in similar ranges as observed in the present study (45). This concentration of the molecule in circulation is attainable by dietary consumption of a green tea extract supplements (61). Moreover, EGCG has an antioxidant and anti-inflammatory effects (62–64). Of note, one case of resistance to Remdesivir was recently reported in immunocompromised patients just about a year after this drug was approved by the U.S. FDA, justifying the improvement of COVID-19 treatment (65). Therefore, using polyphenols along with Remdesivir could be an option to prevent mild to severe disease or to improve outcomes of patients with severe COVID-19 as well as to limit antiviral resistance.

Remdesivir combined with Isoquercetin showed a synergy score of 11.072. Using an *in vitro* fluorescence resonance energy transfer (FRET) SARS-CoV 3CLpro assay, Ryu et al. (66) have found that quercetin had an IC_{50} of 24 μM, which is in the activity range observed with isoquercetin in our study. Isoquercetin is the glycosylated form of quercetin found in plants. Its absorption is either intact through GLUT2 transporters or after deglycosylation from enterocyte produced lactose phloridzin hydrolase releasing the aglycone in circulation. Absorbed quercetin is then rapidly metabolized by hepatic phase II enzymes, leading to the glucuronidated form of the molecule. The molecule is thus bioavailable and can act systemically. Of note, an open-label randomized phase-2 study of Isoquercetin and standard of care versus standard of care only for the treatment of COVID-19 will be undertaken in 2022 (https://clinicaltrials.gov/ct2/show/record/NCT04536090).

Although the antiviral combinations demonstrated efficacy against SARS-CoV-2, they do not appear to be cytotoxic. Antiviral cytotoxicity was evaluated alone or in combination. Indeed, no cytotoxic effect was observed with all antiviral concentrations tested (**Figures 2A, B**). This observation confirms the low cytotoxic concentration 50 (CC50) of Quercetin observed previously (67).

CONCLUSION

This study confirmed the inhibitory effects of two polyphenols, EGCG and Isoquercetin, against SARS-CoV-2 and demonstrated their antiviral synergistic effects with Remdesivir *in vitro*. In order to improve antiviral therapy, to reduce the development of resistant strains or intolerance by using a high dose of Remdesivir alone, this study demonstrated that combining polyphenols and Remdesivir, which have different mechanisms of action, could be considered as an efficient therapeutic alternative. Also, EGCG and Isoquercetin could be used for prophylaxis in high-risk populations, or for the

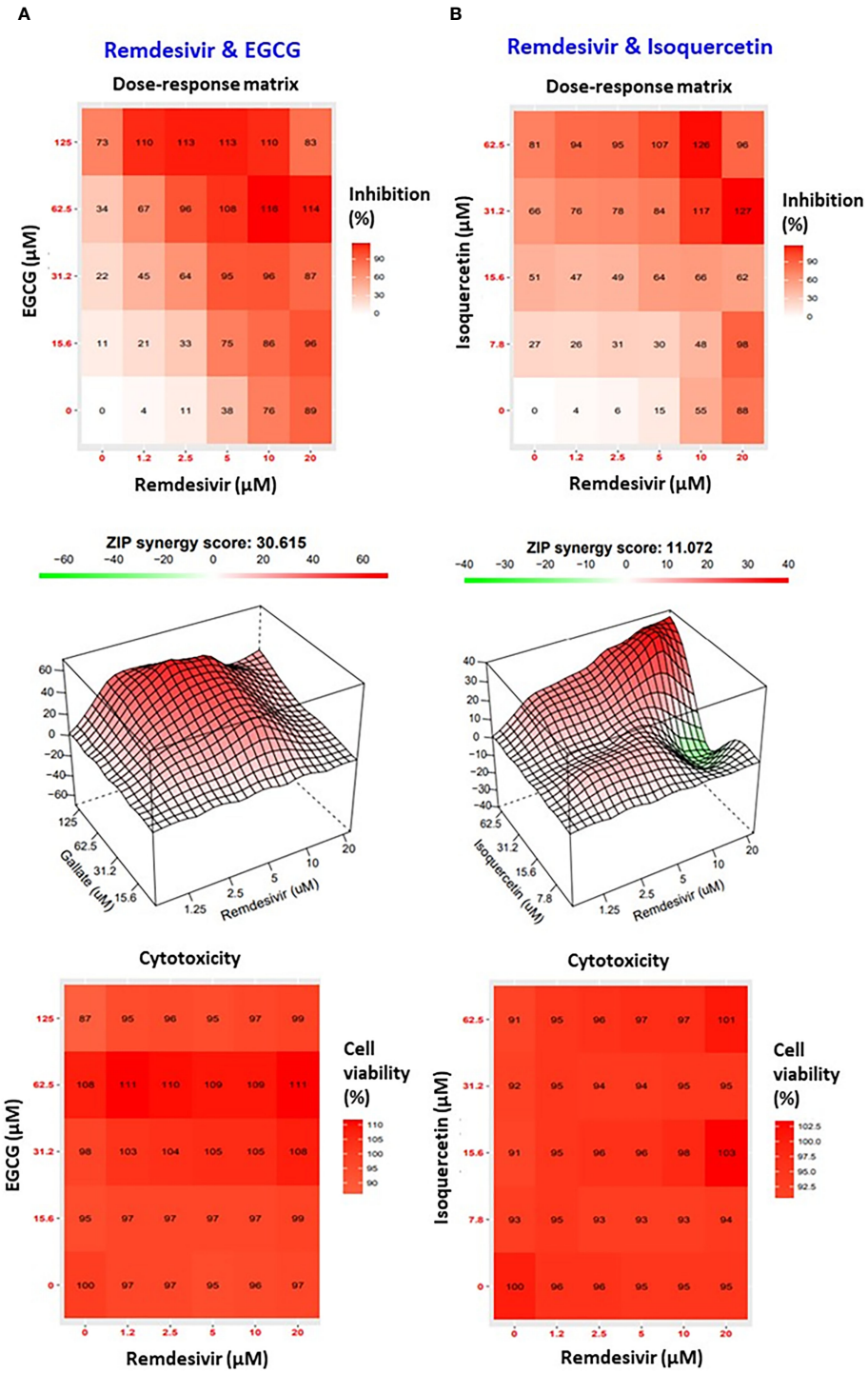

FIGURE 2 | Synergistic antiviral effects of polyphenols and Remdesivir against SARS-CoV-2. **(A)** Combination of Remdesivir- EGCG. At the top, dose-response matrix with the percentage of inhibition induced by single and combination drugs on SARS-CoV-2 infected cells (red box=high percentage of virus inhibition); In the middle, interaction surface response between these compounds with the synergy score for the combination using ZIP reference model (red areas correspond to a synergy score larger than 10 = synergistic interaction between two drugs); At the bottom, cytotoxicity evaluation on uninfected cells (red box=high percentage of cell viability) of **(A)** Remdesivir-EGCG and **(B)** Remdesivir-Isoquercetin.

treatment of COVID-19 patients as an adjunct to Remdesivir due to their strong synergistic effect against SARS-CoV-2, especially for EGCG which has shown the highest synergy score. Moreover, if an orally bioavailable formulation of Remdesivir shows promising results, this combination could be extended to outpatients. Our results warrant further studies to explore the efficacy of each combination therapy using animal models as well as clinical investigations for the prophylaxis and treatment of SARS-CoV-2 infections.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

Conceptualization, HR and MB; methodology, HR, AB, LC, KP, and MB; software, HR and LC; validation, YD and MB; formal analysis, HR and MB; supervision, YD and MB; writing—original draft preparation, HR; writing—review and editing, YD and MB; funding acquisition, YD and MB. All authors have read and agreed to the published version of the manuscript.

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