Research Article

Türk Fen ve Sağlık Dergisi
Turkish Journal of Science and Health

Volume 2
Number 3
Year 2021
Pages 24-34

e-ISSN: 2717-7173 https://dergipark.org.tr/tr/pub/tfsd

Received: 20.08.2021 *Accepted:* 08.09.2021 https://doi.org/10.51972/tfsd.983961

Antiviral Effects of Some Flavonoids on SARS-CoV-2

Aynur Müdüroğlu Kırmızıbekmez [1]*, Cihan Mehmet Altıntaş [2], Ali Arslan [2], İhsan Kara [2]

[1]Department of School of Health Science, Nişantaşı University, İstanbul, Turkey
[2]Sankara Brain and Biotechnology Research Center, İstanbul University Cerrahpasa Avcılar Campus, Avcılar, İstanbul

ABSTRACT:
Purpose: Covid-19 disease caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is a global epidemic that affects millions of lives. To date, there is no definitive cure for the disease and global vaccination efforts with newly produced vaccines will take years to complete. *In silico* studies have suggested that different flavonoids play an antiviral role against SARS-CoV-2. In this study, based on *in silico* findings, we examined the *in vitro* effects of four promising flavonoids, hesperidin, oleuropein, epigallocatechin gallate (EGCG), and myricetin.
Material and Methods: Hesperidin, oleuropein, EGCG, and myricetin have been extracted from natural plant sources and purified by using liquid chromatography (LC). Analyses of the cell toxicity and antiviral activity of these four flavonoids at different concentrations against SARS-CoV-2 were done.
Results: Our results show that CC_{50} values for EGCG, hesperidin, myricetin and oleuropein are 38 µg/ml, 25 µg/ml, >200 µg/ml and >200 µg/ml, respectively. In addition, in our hands, neither of the flavonoids we examined has antiviral effects against Sars-CoV-2 virus-infected Vero E6 cells. Our data revealed that all flavonoids we tested have Inhibitory Concentration 50 (IC_{50}) value >200 µg/ml.
Conclusion: Our results using the SARS-CoV-2 infected Vero E6 cell model were found to contradict previous *in silico* findings, and these flavonoids were found to have no antiviral effects *in vitro*. Studies investigating flavonoids' antiviral activity on SARS-CoV-2 should be directed to those other than oleuropein, hesperidin, EGCG, and myricetin.
Keywords: *Covid-19, EGCG, Hesperidin, Myricetin, Oleuropein*

**Corresponding author: Aynur Müdüroğlu Kırmızıbekmez, email: aynur.muduroglu@nisantasi.edu.tr*

INTRODUCTION

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) pandemic was first reported in Wuhan in the Hubai district of the Republic of China on 31st December 2019 (Guan et al., 2020). The disease SARS-CoV-2 causes were named Covid-19. Coronaviridae Study *Group* (CSG) of the *International Committee* on *Taxonomy* of *Viruses* (ICTV) suggested classifying this isolated new type of virus as the 7th member of the coronavirus family and named SARS-CoV-2 (Coronaviridae Study Group, 2020). Members of the coronavirus family are zoonotic. They are a large family of infectious viruses that can spread from animals to humans (Zhu et al., 2020a). SARS-CoV-2 genome was reported to have 86-89% similarity with two bat SARS-like-coronavirus isolate bat-SL-CoVZC45 genomes. It also has a 79% similarity with SARS-CoV and 50% with MERS-CoV genomes. Moreover, other genomic studies revealed that the SARS-CoV-2 genome has a 96% similarity with the Bat-Cov RaTG13 isolate genome isolated from the bat species *Rhinolophus affinis* (Lu et al., 2020). It has been found that ACE2 receptors are related to SARS-CoV-2 and are the entry point of the virus (Bastolla, 2020). SARS-CoV and SARS-CoV-2 bind to the target epithelial cells expressing

angiotensin-converting enzyme 2 (ACE2) in lungs, blood vessels, kidneys, and intestines. ACE2 expression can be increased by using ibuprofen and thiazolidinediones. Increased ACE2 expression makes getting Covid-19 infection easier (Fang et al., 2020). Thus, diabetes and hypertension that are treated with such drugs may increase the risk of developing severe and lethal Covid-19 symptoms. To our knowledge, no antiviral medicine has been developed against MERS-CoV, SARS-CoV, and SARS-CoV-2, to date. So far, drugs such as Hydroxychloroquine (HCQ), Chloroquine (CQ), Favipiravir, Lopinavir/Ritonavir (LPV/r), Nitazoxanide, Tocilizumab, Ivermectin, Dexamethasone (DEX) have been used in different treatment regimens to treat Covid-19 patients (Iyer et al., 2020). According to the World Health Organization (WHO) report on 17.08.2021, there are 207784507 confirmed cases and 4370424 deaths worldwide. Turkey's first case was reported on 11.03.2020, and there are 6096816 confirmed cases and 53327 deaths to date. Currently, there is neither definite treatment nor cure for Covid-19. Moreover, current treatments are insufficient for preventing the long-term effects of Covid-19 on further complications and organ damages (Rodriguez-Morales et al., 2020). Some preliminary studies examined the effects of the potential lopinavir/ritonavir combination, commonly used for treating human immunodeficiency virus (HIV) patients, for Covid-19 treatment (Lu, 2020).

Flavonoids are shown to have antiviral effects on various viruses such as Poliovirus (Conti et al., 1990), Astrovirus (Superti et al.,1990), HIV (Clercq, 2000), Enterovirus (Genovese et al., 1995), parainfluenza virus type 3 (PIV 3), respiratory syncytial virus (RSV), and influenza virus type A (Flu A) Studies on flavonoids have shown that luteolin, apigenin, amentoflavone, quercetin (Ryu et al., 2010), puerarin, daidzein, gallocatechin gallate, and epigallocatechin gallate inhibit the original SARS-CoV 3CLpro's proteolytic activity (Nguyen et al., 2012). Green tea catechins (GTC) are the polyphenolic compounds extracted from the leaves of *Camellia sinensis* (Narotzki et al., 2012). These polyphenol-rich compounds have anti-inflammation (González et al., 2015), anti-oxidative (Chacko et al., 2010), anti-bacterial (Umashankar et al., 2018), and antiviral (Ciesek et al., 2011) properties. GTCs mainly comprise four compounds: Epigallocatechin (EGC), Epicatechin (EC), Epigallocatechin gallate (EGCG), and Epicatechin gallate (ECG). Among these, EGCG comprises 59% of the total polyphenol of GTCs and the most complex and major component that has antiviral activity against hepatitis C virus (Ciesek et al., 2011), human immunodeficiency virus (HIV) (Yamaguchi et al., 2002), Zika virus (Carneiro et al., 2016), Influenza A virus (Zhu et al., 2020b) and herpes simplex virus 1 (Isaacs et al., 2008). The antiviral mechanism of EGCG varies depending on specific virus infection and host cell response. For example, one of its antiviral mechanisms is that EGCG interacts with the virus's structural proteins and thus inhibits the recognition and binding of the cellular receptors (Li et al., 2020). Thousands of plant species synthesize phenolic compounds such as phenolic acids, phenolic alcohols, and flavonoids. However, secoiridoids containing many coumarin-like compounds synthesized only by Oleaceae family plants, including *Olea europea L*. (Silva et al., 2016). Oleuropein is the most significant of these compounds. Oleuropein belongs to the secoiridoids abundantly found in Oleaceae, Gentianaceae, Cornaleae, and some other plants. Iridoids and secoiridoids are usually glycosidic bound and produced in the secondary metabolisms of terpenes as indol and alkaloid primers (Omar, 2010). Oleuropein and derivatives have anti-inflammatory, antioxidant, antiviral, antimicrobial, and anti-proliferative properties. They also have protective effects on diabetes mellitus, cardiovascular disease, neurological diseases, cancers, and skin problems (Rigacci and Stefani, 2016).

In their *in silico* docking study of 4634 effective compounds, Leif Peterson found that flavonoids group Diosmin, epigallocatechin gallate, and hidrosmin placed 22nd, 134th, and 163rd, respectively (Peterson, 2020). In another *in silico* study, Khaerunnisa et al. suggested that drugs such as lopinavir and nelfinavir might represent a potential therapy, and luteolin-7-glucoside dimethoxy curcumin, oleuropein, apigenin-7-glucoside, curcumin, catechine, and epicatechin-gallate could be used as Covid-19 M^{pro} inhibitors (Khaerunnisa et

al., 2020). Along with quercetin and kaempferol, myricetin (3,3,4,5,5,7-hexahydroxyflavone) is a member of the flavonoid group flavonols. Various studies revealed that myricetin has antioxidant (Mendes et al., 2019) and anti-inflammatory (Wang et al., 2010) properties as well as anti-bacterial (Xu and Lee, 2001) and antiviral (Yu et al., 2012) effects. Hesperidin is one of the most important flavonoids and belongs to the flavanone class. Hesperidin (hesperetin-7-O-rutinoside) is the β-glycoside form of hesperetin and is found in citrus, especially in sweet orange (Boonpawa et al., 2017). Hesperidin has a strong antioxidant activity and important effects on the vascular system by decreasing capillary permeability and increasing its resistance. Hesperidin has also been shown to decrease cholesterol and triglyceride levels significantly and has remarkable protective effects against inflammatory diseases (Meneguzzo et al., 2020). In their recent *in silico* study, Chen et al. scanned 1500 potential substances based on 3CLpro structure. Among their results, diosmin was placed first, and hesperidin was placed second in the biflavonoid group (Chen et al., 2020). In another recent study, 80 flavonoids were scanned, and several flavonoids with various properties such as hesperidin, rutin, diosmin, apiin, diacetycurcumin, myricetin, flavone23, naringin, neohesperidin, and scutellarin could be used as an alternative therapy for SARS-Cov-2 (Adem et al., 2020). Considering the promising results of the *in silico* studies, the potential therapeutic and prophylactic effects of plant-isolated natural flavonoids should be further investigated by *in vitro* studies to reveal their efficiency. Therefore, in this study, we aimed to purify natural flavonoids EGCG, Oleuropein, Hesperidin, and Myricetin from plants and examine their antiviral effects against SARS-CoV2.

MATERIAL and METHODS

Chemicals and Standards

Acetonitrile (HPLC grade, >99.8%), formic acid (pro analysis, 98−100%), and methanol (HPLC grade, >99.8%) were obtained from ISOLAB Chemicals (Eschau, Germany). Oleuropein, hesperidin (Hesperitin 7-rutinoside), and myricitrin (Myricetin 3-O-rhamnoside) were purchased from Sigma (St. Louis, MO) and EGCG was purchased from TOCRIS Bioscience (Bristol, UK). Ultrapure water was obtained by use of Direct-Q 3 UV, Milli-Q® (Darmstadt, Germany).

Extraction of Oleuropein Compound

Extraction of oleuropein from olive leaves was performed using Cifa et al. method with some modifications (Cifá et al., 2018). Olive leaves collected from the Çanakkale region in Turkey were first washed and dried at 40°C in drying ovens. Then the dried leaves were powdered by using a laboratory mill (IKA A10 basic) and ultrasonicated using 1:10 pure water at 45°C for 60 minutes. Following this step, the liquid phase was collected using filtering and centrifuging at 5000 rpm for 10 minutes. Finally, the upper phase was analyzed using HPLC.

HPLC-DAD Analysis of Oleuropein

HPLC analysis of oleuropein was performed using Aouidi et al. method with some modifications (Aouidi et al., 2012).

Shimadzu LC-20A (Kyoto, Japan) was used for HPLC analyses. This system comprises a PDA detector (SPD-M20A) and GL Sciences Inertsil ODS-3 C18 (250 x 4.6 mm; 5 µm) HPLC column. The pre-analysis sample was filtered through a 0.45 µm filter. In addition, all the mobile phases were filtered before analysis and incubated in an ultrasonic bath for 30 minutes. The sample injection volume was set to 20 µL, and the column oven temperature was set to 25°C. The mobile phase was 5% formic acid in water (A) and acetonitrile (B) with a flow rate at 0.9 ml/min in gradient (Table 1). Chromatograms were recorded using a PDA detector set to 280 nm. All the calculations concerning the quantitative analysis were performed with oleuropein standard by matching the retention times and measurement of peak areas. Data were analyzed using LC solution software.

Extraction of EGCG Compound

Green tea leaves were obtained from the Black Sea region in Turkey. Extraction of catechins from green tea leaves was performed using Lee and Lee method (Lee and Lee, 2008). First, green tea leaves were

washed and dried at 30°C in drying ovens. Then, the dried leaves were powdered by using IKA A10 basic mill up to 100 mesh size. Powdered leaves were extracted in pure water (1:10) using an ultrasonication system. The extraction was performed at 60 °C for 30 minutes. Finally, the resulted extracts were centrifuged at 5000 rpm for 10 minutes and upper phase was recovered to be analyzed in HPLC.

Table 1. Oleuropein gradient elution used in method

Time (min.)	B%
0	5
3	15
13	25
25	35
35	45
40	50
45	100
46	5
50	5

Table 2. EGCG gradient elution used in the method

Time (min.)	B%
0	10
5	15
50	40
51	10
55	10

Table 3. Myricetin gradient elution used in the method

Time (min.)	B%
10	13
20	41,5
25	70
35	10
36	0
40	0

Table 4. Hesperidin gradient elution used in the method

Time (min.)	B%
0	15
25	35
27	70
35	70
40	15
45	15

HPLC-DAD Analysis of EGCG

Catechine content analysis of green tea leaves was performed using Dalluge et al. HPLC method with some modifications (Dalluge et al., 1998). Shimadzu LC-20A (Kyoto, Japan) was used for HPLC analyses. This system comprises a PDA detector (SPD-M20A) and GL Sciences Inertsil ODS-3 C18 (250 x 4.6 mm; 5 µm) HPLC column. The pre-analysis sample was

filtered through a 0.45 µm filter. In addition, all the mobile phases were filtered before analysis and incubated in an ultrasonic bath for 30 minutes. The sample injection volume was set to 20 µL, and the column oven temperature was set to 25°C. The mobile phase was 0.05% trifluoroacetic acid (TFA) in water (A) and 0.05% TFA in acetonitrile – methanol (40:60) (B) with a flow rate at 1.0 ml/min in gradient Table 2). Chromatograms were recorded using a PDA detector set to 210 nm. All the calculations concerning the quantitative analysis were performed with EGCG standard by matching the retention times and measurement of peak areas. Data were analyzed using LC solution software.

Extraction of Myricetin Compound

Leaves of bilberry (*Vaccinium myrtillus*) for myricetin derivatives were obtained from a local market in Malatya, Turkey. They are originated from the southeast region of Turkey. Myricetin-rich extraction from bilberry leaves was performed using Lui et al. method (Liu et al., 2014). First, the leaves were thoroughly washed and dried in drying ovens at 40°C. Dried leaves were then powdered by using a laboratory mill (IKA A10 basic) and extracted in pure water (1:10) using an ultrasonication system. The extraction was performed at 45°C for 60 minutes. After extraction, samples were centrifuged at 5000 rpm for 10 minutes and the upper phase was collected for analysis in HPLC.

HPLC-DAD Analysis of Myricetin Compound

HPLC analysis of phenolic substances in bilberry leaves was done using Sahin et al. method with some modifications (Sahin et al., 2011). HPLC analysis of hesperidin was done using Fecka and Turek method with some modifications (Fecka and Turek, 2007). Shimadzu LC-20A (Kyoto, Japan) was used for HPLC analyses. This system comprises a PDA detector (SPD-M20A) and GL Sciences Inertsil ODS-3 C18 (250 x 4.6 mm; 5 µm) HPLC column. The sample injection volume was set to 20 µL, and the column oven temperature was set to 25 °C. The mobile phase was 1% formic acid in water (A) and acetonitrile (B) with a flow rate of 1.0 ml/min in gradient (Table 3). Chromatograms were recorded using a PDA detector set to 320 nm. All the mobile phases were filtered before analysis and incubated in an ultrasonic bath for 30 minutes to degas. Data were analyzed using LC solution software.

Purification of Oleuropein, EGCG, and Myricetin by Using Prep-LC

Agilent 1260 Infinity II preparative LC system was used for the purification of oleuropein, EGCG, and myricetin. This system comprises a pump with a maximum of 50 ml/min flow rate, UV detector, fraction collector, and manual sampling unit with various injection volumes. To obtain oleuropein, EGCG, and myricetin in high purity, we first adjusted their analytical HPLC methods to the preparative LC system by the scale-up method. Obtained fractions were dried using a nitrogen evaporator. Purity analysis of the fractions was performed using analytical HPLC.

Extraction and Purification Method for Hesperidin

Peels of orange (*Citrus sinensis*) for hesperidin were obtained from the wastes of the juice plant in the Mediterranean region, Turkey. To perform dried experiments, orange peels were placed in an oven at 40°C overnight until constant weight. Hesperidin from sweet orange peels was extracted using Lahmer et al.'s method with some modifications (Lahmer et al., 2015). First, orange peels were washed and placed in drying ovens at 40°C to dry. This step is followed by powdering the peels in a laboratory mill (IKA A10 basic) and extracting them using 1:10 petroleum ether for 90 minutes. After removing the petroleum ether, the dry pellet was mixed with methanol for secondary extraction and ultrasonicated for 60 minutes at 45 °C. Following this procedure, the liquid phase was collected using filtering and centrifuging, and methanol was removed in a rotary evaporator. Lastly, the condensed phase was dissolved in 6% acetic acid to precipitate hesperidin. After centrifugation at 5000 rpm for 15 minutes, the pellet was collected and dried. Powder pellet was analyzed for hesperidin percentage using HPLC.

HPLC-DAD Analysis of Hesperidin

HPLC analysis of hesperidin was done using Fecka and Turek method with some modifications (Fecka

and Turek, 2007). Shimadzu LC-20A (Kyoto, Japan) was used for HPLC analyses. This system comprises a PDA detector (SPD-M20A) and GL Sciences Inertsil ODS-3 C18 (250 x 4.6 mm; 5 µm) HPLC column. The pre-analysis sample was dissolved in an adequate solvent and filtered through a 0.45 µm filter. In addition, all the mobile phases were filtered before analysis and incubated in an ultrasonic bath for 30 minutes. The sample injection volume was set to 20 µL, and the column oven temperature was set to 25°C. The mobile phase was 5% formic acid in water (A) and acetonitrile (B) with a flow rate at 0.9 ml/min in gradient (Table 4). Chromatograms were recorded using a PDA detector set to 280 nm. All the calculations concerning the quantitative analysis were performed with the hesperidin standard by matching the retention times and measurement of peak areas. Data were analyzed using LC solution software.

Cytotoxicity Test

In this study, we used Vero E6 cells, African green monkey (*Cercopithecus aethiops*) kidney epithelial cells (ATTC, CCL-81). Cells are cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) with 10% Fetal Bovine Serum (FBS)(Gibco). Cell cultures were maintained at 5% CO_2 and 37° C. Cells were prepared as a $2x10^5$/ml suspension and distributed in each well of the 96-well plate at an amount of 100 µl/well. After additional culturing cells for 24 hours at 37° C and 5% CO_2 in 96-well plates, test materials that are diluted in DMEM with 1% FBS and gentamycin/fungizon at a final concentration of 200 - 0.1 µg/ml were added. For each concentration, three replicates (wells) were used. Wells that do not contain test material were used as negative controls. Cells were incubated for 72 hours at 37° C and 5% CO_2. Before analysis, wells including the negative controls were emptied, and 0.1 ml DMEM with 1% FBS and gentamycin/fungizon were freshly added to each well. After incubating for 30 minutes at 37° C and 5% CO_2, 10 µl of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) at a concentration of 5 mg/ml was added to each well and incubated for 3 hours 37° C and 5% CO_2. After the incubation period, cells were emptied, and 100 µl /well dimethyl sulfoxide (DMSO) was added to each well to dissolve intracellular formazan crystals. After 30 minutes of incubation with DMSO, color intensity was analyzed using ELISA Microplate Reader (Tecan, Sunrise) at 570/620 nm wavelength. The percentage of cytotoxicity (%) was calculated using the formula [1-(test material - blank)/(cell control - blank)] x 100. *inhibition* percentages (%) were calculated using the formula (test material - solvent) / (cell control - solvent) X 100. Cells were prepared as a $2x10^5$/ml suspension and distributed in each well of the 96-well plate at an amount of 100 µl/well. Cell cytotoxicity 50 (CC_{50}) and inhibitor concentration 50 (IC_{50}) values were calculated using non-linear regression analysis with GraphPad software. Antiviral activity experiments of this study were done by Antimikrop R&D and Biocidal Analysis Center (Ankara, Turkey).

Antiviral Test

Cells were prepared as a $2x10^5$/ml suspension and distributed in each well of the 96-well plate at an amount of 100 µl/well. After additional culturing cells for 24 hours at 37° C and 5% CO_2 in 96-well plates, cells were infected using 100 TCID50/0.1 ml SARS-CoV-2 (clinical isolate) (GenBank: MT955161.1) in DMEM with %1 FBS for 1 hour at 37° C and 5% CO_2. After the incubation period, viral inoculation was removed, and test materials that are diluted in DMEM with 1% FBS and gentamycin/fungizon at a final concentration of 200 - 0.1 µg/ml were added. For each concentration, three replicates (wells) were used. Wells that do not contain test material were used as negative controls. In addition, eight wells with and without viruses were used as controls. Cells were incubated for 72 hours at 37° C and 5% CO_2. Before analysis, wells including the negative controls were emptied, and 0.1 ml DMEM with 1% FBS and gentamycin/fungizon were freshly added to each well. After incubating for 30 minutes at 37° C and 5% CO_2, 10 µl of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) at a concentration of 5 mg/ml was added to each well and incubated for 3 hours 37° C and 5% CO_2. After the incubation period, cells were emptied, and 100 µl /well dimethyl sulfoxide (DMSO) was added to each well to dissolve intracellular formazan crystals. After 30 minutes of incubation with DMSO, color intensity was analyzed

using ELISA Microplate Reader (Tecan, Sunrise) at 570/620 nm wavelength. Cytopathic and inhibitory effects of each test material at each concentration were analyzed using the following formulas. % inhibition values were calculated using the formula [(Test Optical Density/Control Optical Density)×100]. Test material concentration with 50% inhibitory effect (inhibitory concentration 50, IC_{50}) values were calculated using non-linear dose-response curve: log(inhibitor) vs. normalized response Variable slope with GraphPad software.

RESULTS

Purification of Flavonoids

To examine the antiviral effects of natural flavonoids on SARS-CoV-2 infection we first purified them from respective plants using methods described in the materials-method section. Briefly, we purified Oleuropein from olive leaves originated from the Canakkale region in Turkey, EGCG from green tea leaves originated from Black Sea Region in Turkey, Hesperidin from sweet orange peels originated from Akdeniz Region in Turkey, and Myricetin from the Malatya region in Turkey. To identify their purity, the extracts were analyzed by HPLC. According to HPLC analysis, our EGCG extract has >98% purity, Hesperidin extract has >70% purity, Myricetin extract has >60% purity and Oleuropein extract has >80% purity (Figure 1 A-D).

Antiviral Properties of Flavonoids Against SARS-CoV2

We analyzed the antiviral properties of flavonoids with different concentrations (0.1-200 µg/ml) on Vero E6 cells infected with SARS-COV-2. We performed the experiments in triplicates for each concentration. We also analyzed the cell toxicity 50 (CC_{50}) values of each compound separately (Table 5). Our results show that CC50 values for EGCG, Hesperidin, Myricetin and Oleuropein are 38 µg/ml, 25 µg/ml, >200 µg/ml and >200 µg/ml, respectively (Fig. 2A-D). In addition, in our hands, neither of the flavonoids we examined has antiviral effects against Sars-CoV-2 virus-infected Vero E6 cells. Our data revealed that all flavonoids we tested have Inhibitory Concentration 50 (IC50) value >200 µg/ml (Table 5).

Table 5. IC_{50} and CC_{50} values of flavonoids on SARS-CoV-2 infected Vero E6 cells

Flavonoid Name	CC_{50} (µg/ml)	IC_{50} (µg/ml)
EGCG	38	>200
Hesperidin	25	>200
Myricetin	>200	>200
Oleuropein	>200	>200

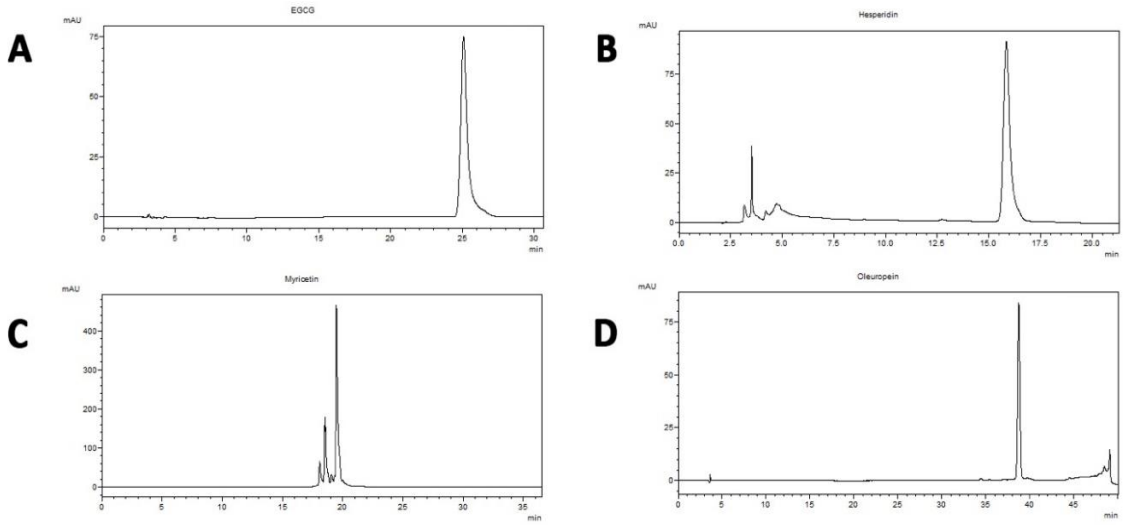

Figure 1 A-D. HPLC chromatogram of A) EGCG extract from green tea leaves acquired at 210 nm detection, B) Hesperidin extract from sweet orange peels acquired at 280 nm detection, C) Myricetin extract from bilberry leaves acquired at 320 nm detection, D) Oleuropein extracts from olive leaves acquired at 280 nm detection.

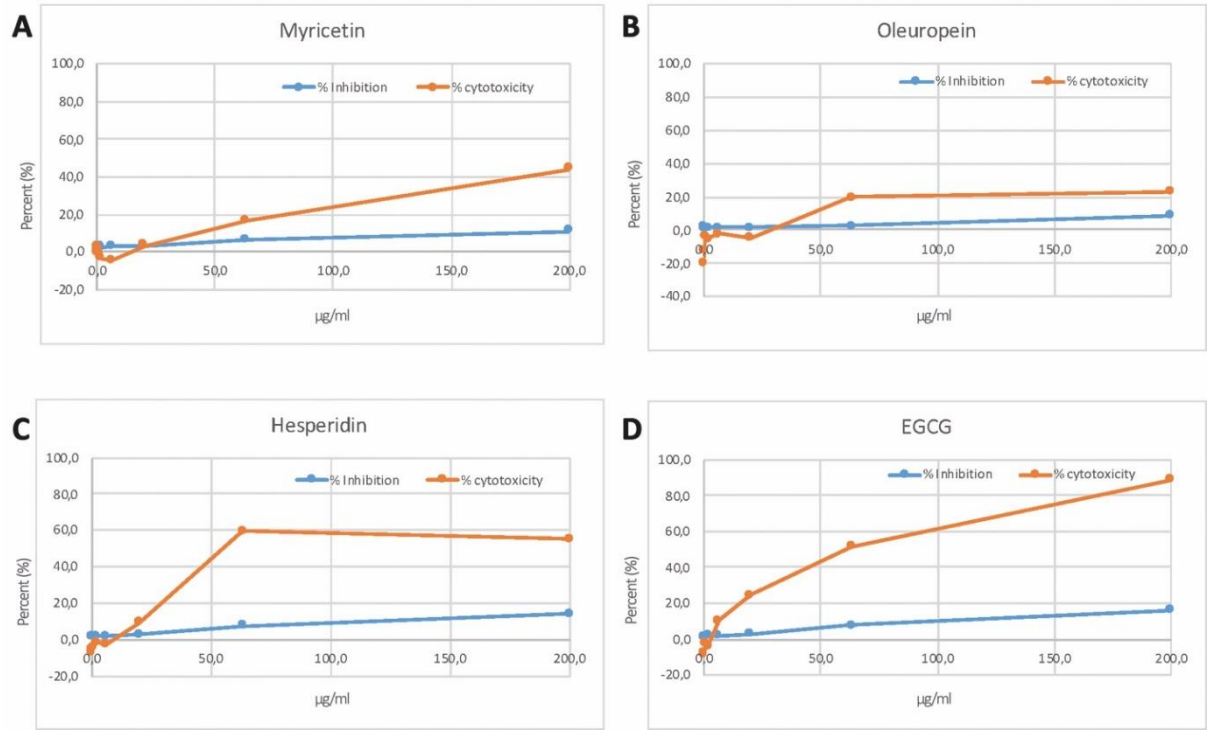

Figure 2 A-D. Antiviral (IC_{50}) and cytotoxic (CC_{50}) values of A) Myricetin, B) Oleuropein, C) Hesperidin D) EGCG on SARS-Cov-2 infected Vero E6 cell.

DISCUSSION

Following the first report in Wuhan, in December 2019, Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) spread worldwide and become a pandemic. The disease SARS-CoV-2 causes was named COVID-19 and has affected millions so far. To date, there is currently no antiviral cure against SARS-CoV-2 and the vaccination of the population with recently developed vaccines will take years. Natural plant-sourced flavonoids are well known for their favorable effects on various viruses such as Poliovuris (Conti et al., 1990), Astrovirus (Superti et al.,1990), HIV (Clercq, 2000), Enterovirus (Genovese et al., 1995), respiratory syncytial virus (RSV), influenza virus type A (Flu A), and parainfluenza virus type 3 (PIV 3) (Wei et al., 2004), and thus, may offer an easily obtainable source for protection from and treatment of COVID-19.

Studies also show that most of the promising small molecules as coronavirus inhibitors are polyphenols that contain a substituted fused ring (Mani et al., 2020). Indeed, flavonoids are well-known for their diverse antiviral effects, and numerous studies have also examined their effect on SARS-CoV-2. Some of these approaches are to increase their bioavailability, and thus, improve their therapeutic efficacy (Ngwa et al., 2020) while others explore the possible use of flavonoids on recovery from COVID-19-Induced anosmia and ageusia (Koyama et al., 2021).

Several *in silico* studies suggested that flavonoids such as EGCG, hesperidin, oleuropein, and myricetin could be used as an alternative therapy for SARS-CoV-2 (Peterson, 2020; Khaerunnisa et al., 2020; Meneguzzo et al., 2020; Adem et al., 2020; Rehman MFu et al., 2021). Considering the promising results of the *in silico* studies, the potential therapeutic and prophylactic effects of plant-isolated natural flavonoids should be further investigated by *in vitro* studies to reveal their efficiency. Thus, in this study, we analyzed the antiviral effects of natural flavonoids EGCG, Oleuropein, Hesperidin, and Myricetin from plants against SARS-CoV2. Although preprint *in silico* studies showed a potential for antiviral effect, our data showed that EGCG, oleuropein, hesperidin and myricetin from plants do not have antiviral effects *in vitro* on SARS-CoV-2 infected Vero E6 cells. There might be several reasons why our *in vitro* results did not support *in silico* findings. First, the interaction of the molecules

in silico assumes an ideal environment. Some other molecules in the culture media as well as the pH and other factors might be interacting either with the docking site or with the compound itself, altering their efficiency and effect. Thus, such possible interactions and alterations may hinder the expected effect of molecules found in ideal conditions *in silico*.

CONCLUSION

Given the worldwide status and severity of SARS-CoV-2, the potential therapeutic and prophylactic effects of natural flavonoids isolated from the plant should be demonstrated by *in vitro* studies to reveal their efficacy. However, flavonoids are strong phytochemicals showing antiviral properties, and these compounds may be toxic in certain concentrations. Therefore, each molecule's safe and therapeutic levels should be tested *in vitro* and in vivo prior to clinical studies in humans. Considering the promising results of *in silico* studies, natural flavonoids EGCG, Oleuropein, Hesperidin, and Myricetin compounds were selected in this study. Our results using the SARS-CoV-2 infected Vero E6 cell model were found to contradict previous in silico findings, and these flavonoids were found to have no antiviral effects *in vitro*. Studies investigating flavonoids' antiviral activity on SARS-CoV-2 *in vitro* should be directed to those other than oleuropein, hesperidin, EGCG, and myricetin. We believe our study will stimulate further studies on the prophylaxis and treatment of coronavirus using other flavonoids and their derivatives.

Acknowledgment

This study was funded by Nişantaşı University Scientific Research Committee (Project No: BAP00017).

Conflict of Interest

The authors declare that there is no conflict of interest.

REFERENCES

Adem, S., Eyupoglu, V., Sarfraz, I. et al. (2020). Identification of Potent COVID-19 Main Protease (Mpro) Inhibitors from Natural Polyphenols: An *in silico* Strategy Unveils a Hope against CORONA. Preprints. doi:10.20944/preprints202003.0333.v1

Aouidi, F., Dupuy, N., Artaud, J. et al. (2012). Rapid quantitative determination of oleuropein in olive leaves (*Olea europaea*) using mid-infrared spectroscopy combined with chemometric analyses. Industrial Crops and Products, 37, 292-297. https://doi.org/10.1016/j.indcrop.2011.12.024

Bastolla, U. (2021). Mathematical model of SARS-Cov-2 propagation versus ACE2 fits COVID-19 lethality across age and sex and predicts that of SARS. Frontiers in Molecular Biosciences, 8, 706122. https://doi.org/10.3389/fmolb.2021.706122

Boonpawa, R., Spenkelink, A., Punt, A. et al. (2017). Physiologically based kinetic modeling of hesperidin metabolism and its use to predict in vivo effective doses in humans. Molecular Nutrition Food Research, 61(8), 1600894. https://doi.org/10.1002/mnfr.201600894

Carneiro, B. M., Batista, M. N., Braga, A. C. S. et al. (2016). The green tea molecule EGCG inhibits Zika virus entry. Virology, 496, 215-218. https://doi.org/10.1016/j.virol.2016.06.012

Chacko, S. M., Thambi, P.T., Kuttan, R. et al. (2010). Beneficial effects of green tea: a literature review. Chinese Medicine, 5, 13. https://doi.org/10.1186/1749-8546-5-13

Chen, Y. W., Yiu, C-P. B., Wong, K-Y. (2020). Prediction of the SARS-CoV-2 (2019-nCoV) 3C-like protease (3CL pro) structure: virtual screening reveals velpatasvir, ledipasvir, and other drug repurposing candidates. F1000Research, 9, 129. https://doi.org/10.12688/f1000research.22457.2

Ciesek, S., Hahn von T., Colpitts, C. et al. (2011). The green tea polyphenol, epigallocatechin-3-gallate, inhibits hepatitis C virus entry. Hepatology, 54(6), 1947-55. https://doi.org/10.1002/hep.24610

Cifá, D., Skrt, M., Pittia, P. et al. (2018). Enhanced yield of oleuropein from olive leaves using ultrasound-assisted extraction. Food Science & Nutrition, 6, 1128-37. https://doi.org/10.1002/fsn3.654

Clercq, E. De. (2000). Current lead natural products for the chemotherapy of human immunodeficiency virus (HIV) infection. Medicinal Research Reviews, 20, 323-349. https://doi.org/10.1002/1098-1128(200009)20:5<323::AID-MED1>3.0.CO;2-A

Conti, C., Genovese, D., Santoro, R. et al. (1990). Activities and mechanisms of action of halogen-substituted flavanoids against poliovirus type 2 infection *in vitro*. Antimicrobial Agents and Chemotherapy, 34(3), 460-466. https://doi.org/10.1128/AAC.34.3.460

Coronaviridae Study Group of the International Committee on Taxonomy of Viruses. (2020). The species severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. Nature Microbiology, 5, 536-544. https://doi.org/10.1038/s41564-020-0695-z

Dalluge, J. J., Nelson, B. C., Thomas, J. B. et al. (1998). Selection of column and gradient elution system for the separation of catechins in green tea using high-performance liquid chromatography. Journal of

Chromatography A, 793(2), 265-74. https://doi.org/10.1016/S0021-9673(97)00906-0

Fang, L., Karakiulakis, G., Roth, M. (2020). Are patients with hypertension and diabetes mellitus at increased risk for COVID-19 infection? The Lancet Respiratory Medicine, 8(4), e21. https://doi.org/10.1016/S2213-2600(20)30116-8

Fecka, I., and Turek, S. (2007). Determination of water-soluble polyphenolic compounds in commercial herbal teas from Lamiaceae: peppermint, melissa, and sage. Journal of Agricultural and Food Chemistry, 55(26), 10908-17. https://doi.org/10.1021/jf072284d

Genovese, D., Conti, C., Tomao, P. et al. (1995). Effect of chloro-, cyano-, and amidino-substituted flavanoids on enterovirus infection *in vitro*. Antiviral Research, 27, 123-136. https://doi.org/10.1016/0166-3542(95)00088-4

González, Y., Torres-Mendoza, D., Jones, G. E. et al. (2015). Marine Diterpenoids as Potential Anti-Inflammatory Agents. Mediators of Inflammation, 2015, 263543-14. https://doi.org/10.1155/2015/263543

Guan, W-J., Ni, Z-Y., Hu, Y. et al. (2020). Clinical Characteristics of Coronavirus Disease 2019 in China. The New England Journal of Medicine, 58(4), 711-712. https://doi.org/10.1016/j.jemermed.2020.04.004

Isaacs, C. E., Wen, G. Y., Xu, W. et al. (2008). Epigallocatechin gallate inactivates clinical isolates of herpes simplex virus. Antimicrobial Agents and Chemotherapy, 52, 962-70. https://doi.org/10.1128/AAC.00825-07

Iyer, M., Jayaramayya, K., Subramaniam, M. D. et al. (2020). COVID-19: an update on diagnostic and therapeutic approaches. BMB Reports, 53(4), 191-205. https://doi.org/10.5483/BMBRep.2020.53.4.080

Khaerunnisa, S., Kurniawan, H., Awaluddin, R. et al. (2020). Potential Inhibitor of COVID-19 Main Protease (Mpro) From Several Medicinal Plant Compounds by Molecular Docking Study. Preprints, 2020030226. Doi:10.20944/preprints202003.0226.v1

Koyama S, Kondo K, Ueha R, Kashiwadani H, Heinbockel T. (2021) Possible Use of Phytochemicals for Recovery from COVID-19-Induced Anosmia and Ageusia. *International Journal of Molecular Sciences*, 22(16), 8912. https://doi.org/10.3390/ijms22168912

Lahmer, N., Belboukhari, N., Cheriti, K. et al. (2015). Hesperidin and Hesperitin preparation and purification from citrus sinensis peels. Der Pharma Chemica, 7, 1-4.

Lee, K. J., and Lee, S. H. (2008). Extraction behavior of caffeine and EGCG from green and black tea. Biotechnology and Bioprocess Engineering, 13, 646-9. https://doi.org/10.1007/s12257-008-0034-3

Li, J., Song, D., Wang, S. et al. (2020). Antiviral Effect of Epigallocatechin Gallate via Impairing Porcine Circovirus Type 2 Attachment to Host Cell Receptor. Viruses, 12(2), 176. https://doi.org/10.3390/v12020176

Liu, P., Lindstedt, A., Markkinen, N. et al. (2014). Characterization of metabolite profiles of leaves of bilberry (*Vaccinium myrtillus* L.) and lingonberry (*Vaccinium vitis-idaea* L.). Journal of Agricultural and Food Chemistry, 62, 12015-26. https://doi.org/10.1021/jf503521m

Lu, H. (2020). Drug treatment options for the 2019-new coronavirus (2019-nCoV). BioScience Trends, 14(1), 69-71. https://doi.org/10.5582/bst.2020.01020

Lu, R., Zhao, X., Li, J. et al. (2020). Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. The Lancet, 395, 565-574. https://doi.org/10.1016/S0140-6736(20)30251-8

Mani, J.S., Johnson, J.B., Steel, J.C. et al. (2020). Natural product-derived phytochemicals as potential agents against coronaviruses: A review. Virus Res., 15, 284. https://doi.org/10.1016/j.virusres.2020.197989

Mendes, R. A., Almeida, S. K. C., Soares, I. N. et al. (2019). Evaluation of the antioxidant potential of myricetin 3-O-α-L-rhamnopyranoside and myricetin 4'-O-α-L-rhamnopyranoside through a computational study. Journal of Molecular Modeling, 25, 89. https://doi.org/10.1007/s00894-019-3959-x

Meneguzzo, F., Ciriminna, R., Zabini, F. et al. (2020). Hydrodynamic Cavitation-based Rapid Expansion of Hesperidin-rich Products from Waste Citrus Peel as a Potential Tool Against COVID-19. Processes 2020, 8, 549. doi:10.20944/preprints202004.0152.v1

Narotzki, B., Reznick, A. Z., Aizenbud, D. et al. (2012). Green tea: a promising natural product in oral health. Archives of Oral Biology, 57(5), 429-35. https://doi.org/10.1016/j.archoralbio.2011.11.017

Ngwa, W., Kumar, R., Thompson, D., Lyerly, W., Moore, R., Reid, T. E., Lowe, H., & Toyang, N. (2020). Potential of Flavonoid-Inspired Phytomedicines against COVID-19. Molecules (Basel, Switzerland), 25(11), 2707. https://doi.org/10.3390/molecules25112707

Nguyen, T. T. H., Woo, H-J., Kang, H-K. et al. (2012). Flavonoid-mediated inhibition of SARS coronavirus 3C-like protease expressed in Pichia pastoris. Biotechnology Letters, 34, 831-8. https://doi.org/10.1007/s10529-011-0845-8

Omar, S. H. (2010). Oleuropein in olive and its pharmacological effects. Scientia Pharmaceutica, 78, 133-54. https://doi.org/10.3797/scipharm.0912-18

Peterson, L. (2020). *İn silico* Molecular Dynamics Docking of Drugs to the Inhibitory Active Site of SARS-CoV-2 Protease and Their Predicted Toxicology and ADME. SSRN Journal, http://dx.doi.org/10.2139/ssrn.3580951

Rehman MFu, Akhter S, Batool AI, Selamoglu Z, Sevindik M et al. (2021) Effectiveness of Natural Antioxidants against SARS-CoV-2? Insights from the *In-Silico* World. *Antibiotics*, 10(8), 1011. https://doi.org/10.3390/antibiotics10081011

Rigacci, S., Stefani, M. (2016). Nutraceutical Properties of Olive Oil Polyphenols. An Itinerary from Cultured Cells through Animal Models to Humans. International Journal of Molecular Science, 17(6), 843. https://doi.org/10.3390/ijms17060843

Rodriguez-Morales, A. J., Bonilla-Aldana, D. K., Tiwari, R. et al. (2020). COVID-19, an Emerging Coronavirus Infection: Current Scenario and Recent Developments - An Overview. Journal of Pure and Applied Microbiology, 14, 05-12. https://doi.org/10.22207/JPAM.14.1.02

Ryu, Y. B., Jeong, H. J., Kim, J. H. et al. (2010). Biflavonoids from Torreya nucifera displaying SARS-CoV 3CLpro inhibition. Bioorganic & Medicinal Chemistry, 18(22), 7940-7. https://doi.org/10.1016/j.bmc.2010.09.035

Sahin, S., Demir, C., Malyer, H. (2011). Determination of phenolic compounds in *Prunella* L. by liquid chromatography-diode array detection. Journal of Pharmaceutical and Biomedical Analysis, 55(5), 1227-30. https://doi.org/10.1016/j.jpba.2011.03.016

Silva, S., Gomes, L., Leitão, F. et al. (2016). Phenolic Compounds and Antioxidant Activity of *Olea europaea* L. Fruits and Leaves. Food Science and Technology International, 12(5), 385-95. https://doi.org/10.1177/1082013206070166

Superti, F., Seganti, L., Orsi, N. et al. (1990). In vitro effect of synthetic flavanoids on astrovirus infection. Antiviral Research, 13, 201-208. https://doi.org/10.1016/0166-3542(90)90038-9

Umashankar, N., Pemmanda, B., Gopkumar, P. et al. (2018). Effectiveness of topical green tea against multidrug-resistant *Staphylococcus aureus* in cases of primary pyoderma: An open controlled trial. Indian Journal of Dermatology, Venereology and Leprology, 84, 163-8. Doi:10.4103/ijdvl.IJDVL_207_16

Wang, S-J., Tong, Y., Lu, S. et al. (2010). Anti-inflammatory activity of myricetin isolated from Myrica rubra Sieb. et Zucc. leaves. Planta Medica, 76(14), 1492-6. Doi:10.1055/s-0030-1249780

Wei, F., Ma, S-C., Ma, L-Y. et al. (2004). Antiviral flavonoids from the seeds of *Aesculus chinensis*. Journal of Natural Products, 67(4), 650-653. https://doi.org/10.1021/np030470h

Xu, H. X., Lee, S. F. (2001). Activity of plant flavonoids against antibiotic-resistant bacteria. Phytotherapy Research 15, 39-43. https://doi.org/10.1002/1099-1573(200102)15:1<39::AID-PTR684>3.0.CO;2-R

Yamaguchi, K., Honda, M., Ikigai, H. et al. (2002). Inhibitory effects of (-)-epigallocatechin gallate on the life cycle of human immunodeficiency virus type 1 (HIV-1). Antiviral Research, 53, 19-34. https://doi.org/10.1016/S0166-3542(01)00189-9

Yu, M-S., Lee, J., Lee, J. M. et al. (2012). Identification of myricetin and scutellarein as novel chemical inhibitors of the SARS coronavirus helicase, nsP13. Bioorganic & Medicinal Chemistry Letters, 22(12), 4049-4054. https://doi.org/10.1016/j.bmcl.2012.04.081

Zhu, J., Ou, L., Zhou, Y. et al. (2020b). (-)-Epigallocatechin-3-gallate induces interferon-λ2 expression to anti-influenza A virus in human bronchial epithelial cells (BEAS-2B) through p38 MAPK signaling pathway. Journal of Thoracic Disease, 12(3), 989-97. http://dx.doi.org/10.21037/jtd.2020.03.20

Zhu, N., Zhang, D., Wang, W. et al. (2020a). A Novel Coronavirus from Patients with Pneumonia in China, 2019. The New England Journal of Medicine, 382, 727-733. https://www.nejm.org/doi/10.1056/NEJMoa2001017