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# *Investigation of The Inhibition of SARS-CoV-2 Spike RBD and ACE-2 Interaction by Phenolics of Propolis Extracts*

# *SARS-CoV-2 Spike RBD ve ACE-2 Etkileşiminin Propolis Ekstraktındaki Fenoliklerle İnhibisyonunun Araştırılması*

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## Abstract

The molecules that consist of propolis are generally polyphenols, and they have many activities such as antiviral, antibacterial and antifungal activities. In this study, it is aimed to investigate the inhibiting capacity of the interaction between ACE-2 and Spike RBD by propolis samples belonging to three different cities (Trabzon, Kocaeli, Kırıkkkale). After determining the propolis sample exhibiting the highest inhibition effect (Kocaeli-1 propolis), the phenolics within aqueous and ethanolic extracts of propolis sample were identified by RP-HPLC-UV and radical scavenging activities, antioxidant capacities, total flavonoids (TFC), phenolic contents (TPC) were determined. Then, individual assessments of the inhibition effects of each phenolic compound were conducted with Spike S1 (SARS-CoV-2): ACE-2 Inhibitor Screening Colorimetric Assay Kit and supported by in silico docking studies. The substances with the greatest inhibitory effect are; protocathecuic acid, caffeic acid, and *p*-coumaric acid with the inhibition of 62.29%, 58.34%, and 59.20%, respectively. The lowest $IC_{50}$ value of the flavonoids was found to be 0.89 mM with caffeic acid. In silico, in vitro experiments, and MTT analyses conducted have demonstrated that caffeic acid and protocatechuic acid can be utilized as highly active compounds against COVID-19.

**Keywords:** Inhibition, Propolis, Protocatechuic acid, SARS-CoV-2

**Özet**

Propolisi oluşturan moleküller genel olarak polifenollerdir ve antiviral, antibakteriyel ve antifungal aktivite gibi birçok aktiviteye sahiptirler. Bu çalışmada, Trabzon, Kocaeli ve Kırıkkale şehirlerine ait propolis örneklerinin ACE-2 ve Spike RBD etkileşimini inhibe etme kapasitesi araştırıldı. Kocaeli-1 propolisinin en yüksek inhibisyon etkisini gösterdiği belirlendikten sonra, propolis örneğinin sulu ve etanolik ekstraktlarındaki fenolik bileşikler RP-HPLC-UV ile tanımlandı ve DPPH radikal temizleme aktiviteleri, antioksidan kapasiteleri, toplam flavonoid (TFC) ve fenolik madde içerikleri (TPC) belirlendi. Daha sonra, her bir fenolik bileşiğin Spike S1 (SARS-CoV-2): ACE-2 İnhibitör Tarama Kolorimetrik Test Kiti ile inhibisyon etkileri bireysel olarak değerlendirildi ve in siliko doklama çalışmalarıyla desteklendi. İnhibitör etkisi en fazla olan maddelerin; protokatekuik asit, kafeik asit, p-kumarik asit olduğu ve bu maddelerin sırasıyla, %62,29, %58,34, %59,20 oranında inhibisyon etkisi gösterdiği belirlendi. Test edilen flavonoidlerden en düşük $IC_{50}$ değerine, kafeik asitin (0,89 mM) sahip olduğu belirlendi. Yapılan in siliko, in vitro deneyler ve literatürdeki MTT analizleri, kafeik asit ve protokatekuik asidin, COVID-19'a karşı oldukça aktif bir bileşik olarak kullanılabileceğini göstermektedir.

**Anahtar Kelimeler:** İnhibisyon, propolis, protokatekuik asit, SARS-CoV-2

**Abbreviations:** ACE-2, Angiotensin Converting Enzyme 2; Spike RBD, Spike receptor-binding domain; MTT, (3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyltetrazolium Bromide)

## 1. INTRODUCTION

Propolis (bee gum) is the resinous substance that honey bees collect from plant buds and shoots, transform it with some enzymes they secrete, and store in the hives. Although propolis varies in structure and composition from region to region and according to flora, it consists of approximately 50-60% resin, 10% wax (wax), and 30-40% balsam. Bees use propolis to protect their hive (colony) as both a physical and chemical defense tool. Propolis is a bee product that has been known and used by humans for a very long time (Kuropatrichi et al., 2013; Lotfy, 2006). Propolis has antibacterial, anti-inflammatory, antiviral (Sforcin et al., 2017), antioxidant, antiprotozoal, anesthetic, antitumoral, anti-cancer, antifungal (Rajpara et al., 2009; Sforcin, 2016), antiseptic, anti-mutagenic properties. It is also used as an anti-hepatotoxic (Toreti et al., 2013). Propolis molecules predominantly comprise polyphenols, exhibiting diverse biological activities including antiviral, antibacterial, and antifungal properties. The antiviral effects of these different polyphenols have been determined on various viruses, such as Coronaviruses, Herpes Simplex viruses, Influenza, Rotaviruses, and Human Immunodeficiency Virus (HIV) (Yıldırım et al., 2019).

Coronaviruses are viruses that were first discovered in the 1960s and are found in birds and mammals, especially bats, cats, camels, and mice (Woo et al., 2012). Coronaviruses are a large family of enveloped, positive-sense, single-stranded RNA viruses with a 5' cap and a 3' polyadenyl tail (Lai & Cavanagh, 1997). The virus that causes COVID-19, has a single-stranded positive-sense RNA genome of approximately 30 kb, which is 74% similar to pangolin (*Manis javanica*) coronaviruses and horseshoe bat (*Rhinolophus sinicus*) coronaviruses (Bat-CoV-RaTG13*)* is 99% (Zhu et al., 2020). It is known that the coronavirus obtained from Malayan pangolins is 99% similar to SARS-CoV-2. There is a single amino acid difference between the Receptor Binding Domain (RBD) of the spike protein of Malayan pangolin coronaviruses and the RBD of SARS-CoV-2. Malayan pangolins infected with this virus also show similar effects to COVID-19 symptoms. Antibodies obtained from infected Malayan pangolins can react with the spike protein of SARS-CoV-2. Although RaTG13 coronaviruses isolated from bats are 96% similar to SARS-CoV-2, the RBDs of two spike proteins are differ from each other, and the binding affinity of the RBD of RaTG13 to the human Angiotensin Converting Enzyme 2 (ACE-2) receptor is low. Six critical amino acids in the receptor binding domain of SARS-CoV-2 and pangolin COV are identical. Considering all these situations, it is suggested that SARS-CoV-2 emerged as a result of the recombination of pangolin-COV and bat-COV-RaTG13 virus. Therefore, the intermediary host between humans and bats is thought to be the pangolin (Liu et al., 2020; Andersen, 2020). The absence of effective prophylactic or therapeutic agent options against viral infections remains a significant issue.

Coronaviruses have four different structural proteins whose functions are fully known and these are spike (S), envelope (E), membrane (M), and nucleocapsid (N). Additionally, several structural proteins are expressed in the viral genome whose exact function is unknown (Lai & Cavanagh, 1997). Among these proteins, the S protein is of great importance for adhesion, fusion, and entry of the virus into the cell, and thanks to these properties, it is seen as an important target for the development of antibodies, entry inhibitors, and vaccines. The spike protein, which protrudes from the virion's envelope, plays an important role in the host receptor selectivity and adhesion to cells. In most coronaviruses, the S protein is cleaved by host proteases into two functional subunits (S1 and S2) of approximately the same size. The N-terminal S1 domain forms the globular head of the S protein and this is where the receptor binding domain (RBD) is located. On the other hand, the S2 domain forms the stem (body) of the S protein, which contains the fusion peptide with two heptad repeat regions, the TM region and the cytosolic tail (Fung & Liu, 2018). During adhesion and entry into the cell, the cellular

protease TMPRSS2 cleaves the S1 and S2 domains to separate them. While the attachment of the virus to the host cell is ensured by the receptor binding domain of the S1 subunit of the nascent S protein, the fusion of the virus and host cell membranes is ensured by the S2 subunit. There is strong scientific evidence that SARS-CoV and SARS-CoV-2 interact with ACE-2 as a receptor. In addition, cellular receptors such as the C-type lectin CD209L and DC-S16S, which are effective in the attachment of SARS-CoV viruses to the cell, play secondary roles (Ortega et al., 2020). The interaction between the viral protein and its receptors on cellular membrane is constitutes a critical step in the replication cycle of the virus. Therefore, the efficiency of viral infection is tightly dependent on this process. Many physicochemical factors are associated with protein-protein interactions. These factors are determined by the nature of the amino acids in the proteins that will interact and the type of the chemical interactions that occur between the ligand and the receptor. RNA viruses that infect cells produce more RNA using host cells, and they use it both to protect their own RNA and to produce proteins to infect new cells. These proteins are the main targets for candidate vaccines and drugs to be developed to prevent COVID-19, and these targets include the spike protein of the virus, the main viral proteases that are specific for degrading the polyprotein of the virus (3-chymotrypsin-like protease (3CLpro), main protease papain-like protease) and the RNA-dependent RNA polymerase of the virus are the leading ones The RBDs of SARS-CoV and MERS-CoV viruses recognize different receptors on host cell surfaces. While SARS-CoV recognizes ACE-2 as a receptor, MERS-CoV recognizes dipeptidyl peptidase 4 (DPP4) as a receptor (Taia et al., 2023). Similar to SARS-CoV, the receptor of the S protein in SARS-CoV-2 is ACE-2. Therefore, the RBD of the S protein of SARS-CoV-2 has been identified as the most likely target for the development of virus binding inhibitors, neutralizing antibodies, and vaccines.

ACE-2, an integral membrane protein, is a protein containing HEXXH-E, a conserved zinc binding motif, consisting of 805 amino acids. ACE-2 is a type I transmembrane metallopeptidase with homology to ACE, an important component of the rennin-angiotensin system (RAS) and a target in the treatment of the hypertension (Riordan, 2003). It is mainly expressed in vascular endothelial cells, renal tubular endothelium, and testicular Leydig Cells (Kuba et al., 2010; Jinag et al., 2014). PCR analyses have shown that ACE-2 is also expressed in lung, kidney, and gastrointestinal tract tissues infected with SARS-CoV (Ksiazek et al., 2003; Harmer et al., 2002). The main substrate of ACE-2 is Angiotensin II (Tikellis and Thomas, 2012), and it breaks down this vasoconstrictor substrate to form Angiotensin 1-7 and thus negatively regulates the RAS system (Kuba et al., 2010; Tikellis & Thomas, 2012) and thus

lowers blood pressure through this hydrolysis. ACE-2 has also been shown to exert a protective function in the cardiovascular system and other organs (Kuba et al., 2010). In this way, it has become a promising drug target for the treatment of cardiovascular diseases.

As stated above, the importance of ACE-2 in terms of SARS-CoV-2 infections is that the Spike protein, with which the virus interacts with the cell in order to infect the cell, attaches to the cell via ACE-2. Considering this situation, this receptor has become one of the main targets of therapeutics to be developed against viral infections. Many studies have recently been carried out on which molecules can eliminate the interactions between the cellular ACE-2 receptor and the viral Spike protein in the development of effective therapeutics. For this purpose, mostly structural biology studies are carried out, and the results of the research conducted in the light of these studies are aimed to create an infrastructure for future studies and to guide scientists in the fight against the virus. Target molecules are tried to be determined through in silico experimental docking studies. For this purpose, it is important to screen natural resources that are thought to contain target molecules with inhibitory effects on the interactions between the cellular ACE-2 receptor and the viral Spike protein and to examine them for the desired activity.

Considering these situations, many researchers are investigating various ways of using propolis against this virus, taking into account the antiviral activity of propolis. Generally, considering the time when COVID-19 infections occur, the first steps in developing effective drugs are experimental molecular modeling studies (molecular docking) and many researchers are working in this field. Some of these studies target the RNA-dependent RNA polymerase of the virus, some target the main protease of the virus, and some aim to stop the interactions between the Spike protein of the virus and its cellular receptor, ACE-2.

In this study, inhibition studies were carried out with propolis extracts obtained from three different locations of Turkey (Trabzon, Kocaeli, and Kırklareli) and were examined in terms of inhibiting the interactions between ACE-2 / Spike protein RBD. Total flavonoid content (TFC), total phenolic content (TPC) and DPPH• radical scavenging activities of the best inhibitory extract (Kocaeli-1 propolis) were determined. Then, the phenolic content of this propolis sample was determined by RP-HPLC-UV. The phenolic substances found in high amounts in Kocaeli-1 propolis extracts were examined in terms of inhibiting the interactions between ACE-2 / Spike protein RBD. Following the in vitro study, a detailed docking study was carried out to demonstrate the interactions of the molecules deemed effective with both

molecules (ACE-2, Spike RBD) separately and interactively (ACE-2 / Spike RBD). With this study, the phenolic content of Kocaeli-1 propolis and the inhibition capacities of the phenolic compounds in its content were determined.

## 2. MATERIALS and METHODS

### 2.1. Chemicals and Kits

COVID-19 Spike Protein: The ACE-2 ELISA kit (Cat. No. 79954) was purchased from BPS Bioscience (San Diego, CA, USA). The chemicals used in the study were gallic acid, protocatechic acid, p-OH benzoic acid, catechin, caffeic acid, syringic acid, epicatechin, *p*-coumaric acid, ferulic acid, routine, myricetin, resveratrol, daidzein, luteolin, t-cinnamic acid, hesperetin, chrysin, pinocembrin, caffeic acid phenethyl ester (CAPE), $FeSO_4.7H_2O$, Folin–Ciocalteu's phenol, diethyl ether, ethyl acetate, and acetonitrile were purchased from Sigma-Aldrich (Sigma-Aldrich Chemie, Munich, Germany). Daidzein was obtained from Cayman Chemical (Michigan, USA) and ferric tripyridyltriazine (Fe-III-TPTZ), $FeCl_3$, $CH_3CO_2Na.3H_2O$, acetonitrile were obtained from Merck (Merck, Darmstadt, Germany).

### 2.2. Preparation of Propolis Extracts

Preparation of aqueous extract from propolis was carried out according to the method specified in application number TPE, 2015/04984. In summary, the method consists of four steps: dewaxing process, the extraction process, filtration, and evaporation process.

In the dewaxing process, propolis ground to 1-10 mm in size or collected from propolis traps in the hives was washed with water not exceeding 30°C. Propolis was laid on the sieve whose hole diameter was smaller than the propolis grinding diameter, with a thickness not exceeding 5 mm. The sieve was rolled up. The roll was placed in a container larger than its diameter and filled with pure water or drinking water until it passed the level of the roll. The temperature of the water was kept between 62-65°C. This process was done externally with a thermostat heater or in a temperature-controlled container. The process continued for no more than 5 hours. At the end of the period, the mixture was cooled. Wax and other resins were observed to collect on the surface of the water, and the resulting wax and other resins were removed from the environment. After the removal of wax and resin, the stages carried out in

the process, which we can generalize as the extraction process; The roll was opened, and the waxed propolis was poured into the same water. The temperature of the water was adjusted to 40-45°C, and it was rotated and extracted at this temperature. During this process, the mixture was acidified with any organic acid (citric acid, malic acid, tartaric acid, lactic acid, etc.) and the mixture was rotated in an acidic environment for at least 30 minutes. During this process, the phenolic compounds dissolved in the acidic environment passed into the solution. At the end of this period, the mixture was alkalized with bases (carbonates) and rotated in an alkaline environment for at least 30 minutes. In this process, those dissolved in the alkaline environment went into solution. By reusing the organic acid, the mixture was brought to the previous pH value. The mixture was filtered in the steps of the filtration and evaporation process that took place after the extraction process. In the preparation of ethanolic and water-based glycerol extracts (aqueous), frozen, ground propolis was added to 70-75% ethanol and glycerol, not exceeding 20%. It was shaken in the dark for 24 hours. It was kept in the refrigerator in the dark for 2 days and then filtered. Propolis samples obtained from three different cities (Trabzon, Kocaeli, and Kırklareli) were extracted by this method.

### 2.3. Determination of Total Flavonoids Content (TFC), Total Phenolic Content (TPC) and DPPH• Radical Scavenging Activity

The total flavonoid content (TFC) within aqueous and ethanolic extracts of Kocaeli-1 propolis were performed in accordance with the methodology established by Fukumoto & Mazza (2000). Quercetin was used for the standard calibration curve. The total flavonoid concentration was measured and expressed as mg of quercetin equivalents per g of the sample.

Phenolic substance quantification was conducted using the Folin-Ciocalteu method, which involves a redox reaction where phenolic compounds reduce the Folin-Ciocalteu reagent, converting into their oxidized state. A gallic acid standard was utilized, following the methodology outlined by Singleton & Rossi (1999). The total phenolic content of Kocaeli propolis extracts were calculated with the absorbance values corresponding to the concentration and expressed as mg of gallic acid equivalents per g of the sample.

Radical scavenging activity was assessed based on the reduction in maximum absorbance of the purple-violet commercial DPPH• (2,2-diphenyl-1-picrylhydrazyl) radical at 517 nm in the presence of the antioxidant material. Concentrations corresponding to the observed absorbances were plotted to calculate $SC_{50}$ values (Cuendet et al., 1997).

### 2.4. Determination of Phenolic Compounds by RP-HPLC-UV

RP-HPLC-UV analysis was conducted using an HPLC system (Elite LaChrom Hitachi) equipped with a UV-VIS detector, operating at a wavelength range of 280-315 nm. The analysis utilized a reverse-phase C18 column (150 mm x 4.6 mm, 5 µm particle size; Fortis), employing a gradient program consisting of acetonitrile, water, and acetic acid.

### 2.5. Spike S1 (SARS-CoV-2): ACE-2 Inhibition Assay

Spike S1 (SARS-CoV-2): ACE-2 Inhibitor Screening Colorimetric Assay Kit was used as a colorimetric Elisa method (BPS Bioscience, 79954). This commercial kit is based on the measurement of binding between the Spike S1 (SARS-CoV-2) protein and the biotin-labeled human ACE-2 protein. Inhibition effects of extracts obtained from three different propolis of cities (Trabzon, Kocaeli, and Kırklareli), different fractions of these propolis extracts, and pure phenolic molecules known to be present in the relevant propolis samples on SARS CoV- 2 Spike RBD/ACE-2 interaction were determined following the procedure prescribed by the company.

### 2.6. Molecular Docking Studies

Based on the in vitro experiments described, the inhibitory effects of propolis extracts from three different provinces and the flavonoid substances detected in these extracts (analyzed using HPLC) were assessed for their impact on the interaction between SARS-CoV-2 Spike RBD and ACE-2 proteins. In this context, 3D structures of each substance showing inhibitory effects were obtained in SDF format from the PubChem (https://pubchem.ncbi.nlm.nih.gov/) database for use in docking studies. The resulting 3D ligand files were converted to pdb format using Openbabel and Pymole software to obtain 3D structures. Subsequently, these compounds were optimized using the MMFF94s force field within the Avogadro software (Hanwell et al., 2012).

The 3D crystal structures of ACE-2 (PDB ID: 1R4L: Resolution 3.00 Å) and SARS-CoV-2 Spike RBD (PDB: 6YLA: Resolution: 2.42 Å) were retrieved from the protein database ((http://www.rcsb.org/pdb), to be used as receptor proteins in the docking studies. Possible docking modes between the ligands and receptor proteins (SARS-CoV-2 Spike RBD and ACE-2) were determined. It was examined using the Autodock 4.2 (Morris et al., 2009) program and the Lamarckian genetic algorithm was used for the docking simulations. Suitable cavities

for the receptors were determined with the BIOVIA Discovery Studio 2018 program, the x, y and z coordinates were created as 126, 126, and 126, with a grid spacing of 0.375 Å. The Autodock program was set to create a total of 100 Genetic algorithms and the settings for all other parameters were saved as default. The molecular docking results, docking score, and binding affinity of each ligand on the corresponding protein target were determined. Visualization and interpretation of all obtained results were performed with BIOVIA Discovery Studio 2018 software (Dassault Systèmes BIOVIA, 2017) . The docking protocol was validated by eliminating the native inhibitor (CR3022 Fab) from complex (Spike receptor binding domain), re-docking and calculating the root mean square deviation (RMSD).

### 2.7. Statistical analysis

Experiments were performed in triplicate (n = 3) and data presented as mean ± standard deviation (SD). Data presented in figures are average of three parallel experiments and error bars are shown for SD. The statistical assessments were performed using the SPSS Version 20.0 (Statistical Package for the Social Sciences). One-way ANOVA was used to determine the statistical differences in the results. Duncan's multiple comparison test was performed to compare statistical difference between the test results. $p<0.05$ was accepted as the significance level.

## 2. RESULTS and DISCUSSION

Since the interaction between ACE-2 and Spike is a protein-protein interaction, it is known that these interactions are revealed by the methods stated below. Protein–protein interactions (PPIs) are fundamental processes for the reproduction and survival of cells and appear to be excellent targets for the development of inhibitors of host-pathogen interactions and biological processes such as cancer cell proliferation. The isolation of PPI inhibitors is quite difficult. There are several in vitro assay methods for testing PPI inhibitors, but they are generally expensive, cumbersome, and require large amounts of purified proteins. However, there are limited in vivo methods to test small molecule PPI inhibitors. While in vivo techniques such as Yeast 2 hybrid (Y2H) and Yeast 3 hybrid (Y3H) analyzes can be used to reveal protein-protein interactions, in vitro analyzes (outside of living cells) such as pull-down and coimmunoprecipitation are techniques used to reveal protein-protein interactions. However, since the yeast two-hybrid system contains artifacts and coimmunoprecipitation requires cell lysis for analysis, the exact

localization of protein-protein interactions within the cell cannot be determined. In contrast, fluorescence resonance energy transfer (FRET) allows the investigation of protein-protein interactions in situ (at their exact localization in the normally occurring cell).

The binding of ACE-2 protein to SARS-CoV-2 spike S1 protein was examined for propolis samples using the inhibitor screening colorimetric assay kit. The main point of this ELISA test is the high sensitivity of the detection of ACE-2-Biotin protein by Streptavidin-HRP. This technique is based on the binding of the active components of propolis to this SARS-CoV-2 Spike RBD/ACE-2 complex and the inhibition of binding of the second enzyme-labeled antibody to the protein. The presence of enzyme activity (horseradish peroxidase) indicates no binding. According to this method, the propolis sample with the highest inhibition effect was determined as 'Kocaeli-1 propolis'. Since ethanol also showed inhibition on HIV-RT as a negative control, the studies were continued with aqueous extracts of Kocaeli-1 propolis, not ethanol extracts.

Quantitative analyses of aqueous and ethanolic extracts of Kocaeli-1 propolis were conducted to determine the total phenolic and flavonoid contents. All results of these assays performed are summarized in Table 1.

Table 1. TPC, TFC, FRAP and radical scavenging activity of aqueous Kocaeli-1 propolis extract*

| **Propolis sample** | **Total phenolic contents (mgGAE/g)** | **Total Flavonoid contents (mgQE/ g)** | **Total Antioxidant Capacity (FRAP) (µmol$FeSO_4.7H_2O$/g)** | **DPPH• radikal Radical Scavenging Activity (SC50, mg/mL)** |
|---|---|---|---|---|
| **Kocaeli-1 (aqueous extract)** | 7.15±0,56 [a] | 2.30±0,40 [a] | 82.30±2,55 [a] | 0.56±0,10 [a] |
| **Kocaeli-1 (%70 ethanol)** | 146.20±1,20[b] | 32,.30±0,58 [b] | 380.20±3,70 [b] | 0.030±0,001 [b] |

* Lowercase letters indicate statistical difference ($p<0.05$)

The phenolic compound composition of aqueous and ethanolic extracts of Kocaeli-1 propolis were revealed by the RP-HPLC-UV method, and this content is summarized in Table 2.

Table 2. Phenolic composition of Kocaeli-1 propolis sample identified by RP-HPLC-UV

| **Standarts (µg fenolic/g sample)** | **Kocaeli-I (ethanol)** | **Kocaeli-I (aqueous)** |
|---|---|---|
| **Gallic acid** | - | 56,20 |
| **Protocatechuic acid** | - | 240 |
| **p-OH Benzoic acid** | - | 120 |
| **Catechin** | - | - |
| **Caffeic acid** | 2460 | 86 |
| **Galangin** | - | - |
| **Syringic acid** | - | 32 |
| **Epicatechin** | - | - |
| **p- Coumeric acid** | 560 | 74 |
| **Ferulic acid** | - | 28 |
| **Quarcetin** | - | - |
| **Rutin** | 4470 | - |
| **Myricetin** | 789 | - |
| **Resveratrol** | - | - |
| **Tannic acid** | - | - |
| **Daidzein** | 138 | - |
| **Luteolin** | 380 | - |
| **Chlorogenic acid** | - | - |
| **Fisetin** | - | - |
| **t-Cinnamic acid** | 530 | 133 |
| **Hesperetin** | - | - |
| **Chyrisin** | 1290 | 230 |
| **Pinocembrin** | 2560 | 142 |
| **Caffeic acid phenetyl ester (CAPE)** | 638 | - |

-: not determined

Differences in composition and TPC, TFC, FRAP and radical scavenging activity between ethanolic and aqueous forms of propolises attributed to solvent and extraction method. The literature contains numerous studies investigating the antioxidant activity of propolis extractions, with research exploring various extraction methods and solvents. (Chen et al., 2019, Turkut et al., 2019, Keskin & Kolayli, 2019, Trusheva et al., 2007). In broader scientific contexts, various types of propolis extracts such as ethanolic, glycol, supercritical fluid, oil-based, and modified aqueous extracts have been shown to exhibit distinct properties in the literature.

Then, the pure molecules known to be present in the Kocaeli-1 propolis sample (gallic acid, protocathecuic acid, p-OH benzoic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid, t-cinnamic acid, chrysin) were tested against SARS-CoV-2 Spike RBD /ACE-2 and the results were shown in Figure 1.

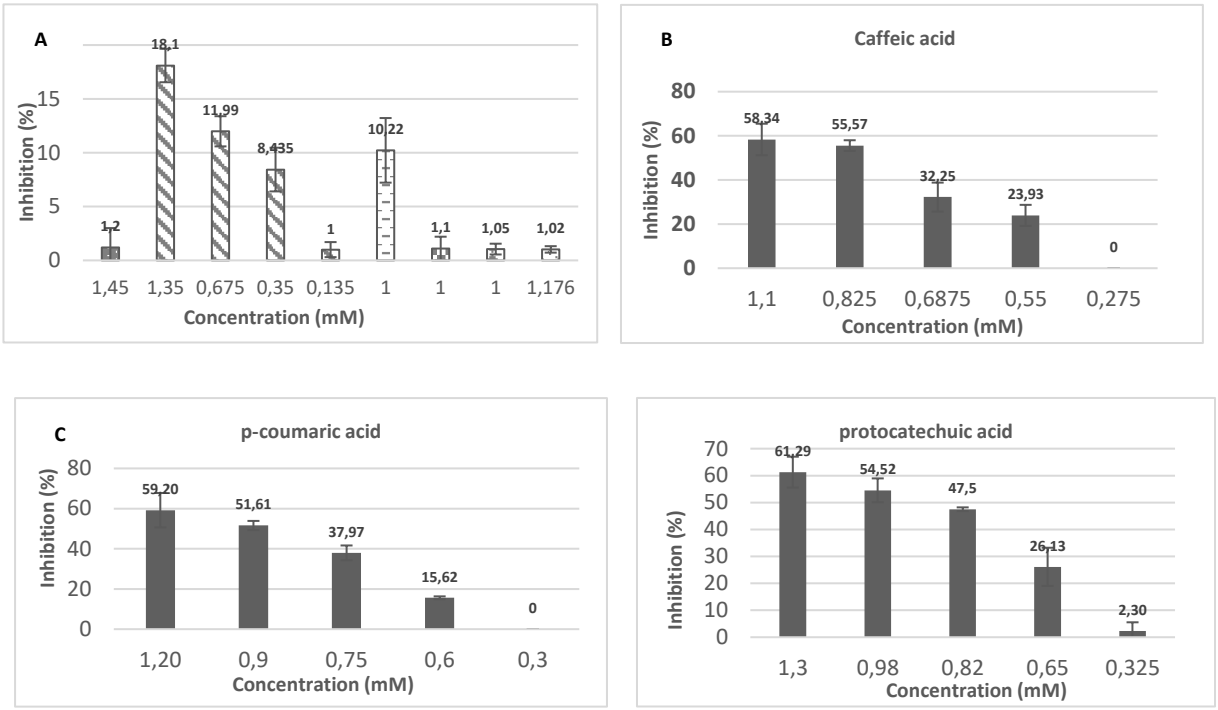


Figure 1. Effect of phenolic compounds in propolis samples on the SARS-CoV-2 Spike RBD/ACE-2 interaction A) *p*-hydroxybenzoic acid, t-cinnamic acid, chrysin, Syringic acid Ferulic acid, Gallic acid B) caffeic acid C) *p*-coumaric acid D) protocatechuic acid

According to the results of preliminary studies, it was determined that *p*-OH benzoic acid, syringic acid, ferulic acid, and gallic acid had no inhibitory effects on the SARS-CoV-2 Spike RBD/ACE-2 interaction. The inhibition values of t-cinnamic acid and chrysin at an average concentration of 1 mM were determined to be 18.1% and 10.22%, respectively (Figure 1A). Among the substances found in the propolis sample, those with the greatest inhibitory effect are; protocathecuic acid, caffeic acid, and *p*-coumaric acid (Figure 1B, 1C, 1D).

The $IC_{50}$ values (half maximal inhibitory concentration) of these substances with the strongest inhibitory effects on the SARS-CoV-2 Spike RBD/ACE-2 interaction were calculated (Table 3).

Table 3. $IC_{50}$ values for protocatechuic acid, caffeic acid and *p*-coumaric acid inhibition of SARS-CoV-2 Spike RBD/ACE-2 interaction

| **Phenolic compound** | **$IC_{50}$ (mM)** |
|---|---|
| **Protocatechuic acid** | 1±0.015 |
| **Caffeic acid** | 0.89±0.016 |
| ***p*-coumaric acid** | 0.99±0.019 |

In addition, the inhibitory effects on the SARS-CoV-2 spike RBD/ACE-2 interaction, which occurred when these substances were added together to the reaction, were also determined. Alone, protocatecuic acid at a concentration of 0.65 mM exhibited an inhibition of 26.13%, while caffeic acid at 0.55 mM demonstrated an inhibition of 23.93%. However, when co-administered, the combined inhibition increased significantly to 62.75%. Similarly, protocatecuic acid at 0.65 mM showed an inhibition of 26.13%, while coumaric acid at 0.6 mM displayed an inhibition of 15.62%. When these compounds were applied together, the resultant inhibition was 58.875%. Moreover, coumaric acid alone at 0.6 mM inhibited 15.62%, whereas caffeic acid alone at 0.55 mM inhibited 23.93%; however, when administered in combination, the inhibition rate was increased to 49.875% (Figure 2). These findings indicate that the combined application of these substances yields higher inhibition rates compared to single application.

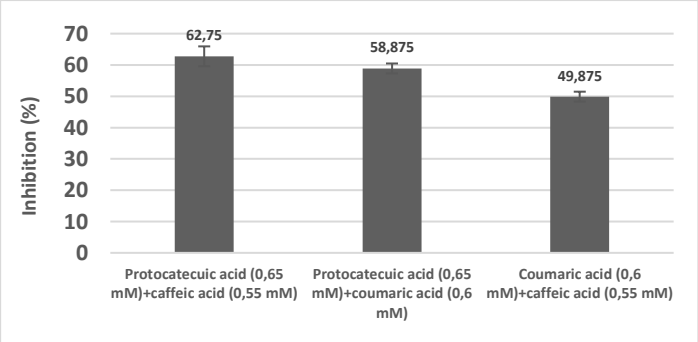


Figure 2. Effect of the combinations of phenolic compounds on the SARS-CoV-2 Spike RBD/ACE-2 interaction

The cytotoxic effects of pure molecules exhibiting the most inhibitory effects on the ACE-2/ Spike RBD interaction were investigated in the literature. In the study conducted by Rezaei-Seresht et al. in 2019; to determine whether caffeic acid is lethal to the cells, using the 3-(4,5-

dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide method, MCF-7 cells (human breast cancer cell line) were treated with different concentrations of caffeic acid ( 5-200 µg/ml) for 48 and 72 hours (MTT assay) and the $IC_{50}$ values were determined. The $IC_{50}$ values of caffeic acid on MCF-7 cells were calculated as 159 µg/ml after 72 hours. The $IC_{50}$ value of caffeic acid used in SARS-CoV-2 Spike RBD / ACE-2 interaction experiments was calculated as 160 µg/ml (0.89 mM) (Rezaei-Seresht et al. 2019). Compared to all other experiments in this study, considering the toxic effects of caffeic acid on breast cancer cells and the morphological changes it causes, the potential of this substance to be used as an antitumor agent in the future is revealed by this study. In another study, *p*-coumaric acid was applied to neuroblastoma N2a cells at concentrations of 1 and 200 µmol/L and kept for 72 hours. The cytotoxic effects of this substance were examined using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide method (MTT assay). According to this; The $IC_{50}$ value was calculated as 104 µmol/L. It was determined that 150 µmol/L *p*-coumaric acid caused 81.23% cell apoptosis after 72 hours (Shailasree et al., 2014). In another study on *p*-coumaric acid; the cell inhibition effects were examined (by MTT assay) on a colon cancer cell line (HT29 and HCT116) and a non-cancer cell line (Vero). *p*-coumaric acid was used at a concentration of 0-3 mM and cells were treated with this agent for 24, 48, and 72 hours. The results show that, the $IC_{50}$ value for HDAC enzymes in the HeLa nuclear extract is 2.6 mM. The antiproliferative effect on the HT29 colon cancer cell line after 72 hours was calculated as $1.0 \pm 0.2$ mM ($IC_{50}$). Again, its antiproliferative effect on the HCT116 colon cancer cell line at the end of 72 hours was calculated as $1.3 \pm 0.8$ mM ($IC_{50}$) (Saenglee et al. 2016). The $IC_{50}$ value of *p*-coumaric acid used in SARS-CoV-2 Spike RBD / ACE-2 interaction experiments was calculated to be 0.99 mM.

In a study conducted for protocatechuic acid, after protocatechuic acid treatment, cell viability was determined by the SRB assay, which is based on the measurement of the ability of SRB to adhere to cell proteins, the total protein amount or the number of cells associated with the SRB dye. After treatment of the cells with protocatechuic acid, incubation was performed for 48 hours. As a result, the $IC_{50}$ values of the cytotoxic effects of protocatechuic acid on MCF-7 and Jurkat cell lines were calculated as $5.97 \pm 0.36$ and $3.15 \pm 0.64$ (mM), respectively. The $IC_{50}$ value of protocatechuic acid used in SARS-CoV-2 Spike RBD / ACE-2 interaction experiments was calculated as 1 mM and is seen to be below this value. According to the studies of Yin et al. 2009; protocatechuic acid has antitumor properties with an effect that increases apoptosis or prevents invasion and metastasis in human breast cancer cell line (MCF-7), lung cancer cell

line (A549), HepG2 cell line, HeLa cell line, cervical cancer cells and LNCaP prostate cancer cells (Yin et al. 2009).

Molecular docking studies were carried out to investigate the inhibitory effects of ligand and reference molecule in silico, that were shown to be effective as a result of the studies carried out with the SARS-CoV-2 Spike RBD / ACE-2 interaction inhibition kit, on the RBD of ACE-2 and SARS-CoV-2 Spike protein. The conformations with high negative binding energy are shown in Figure 3.

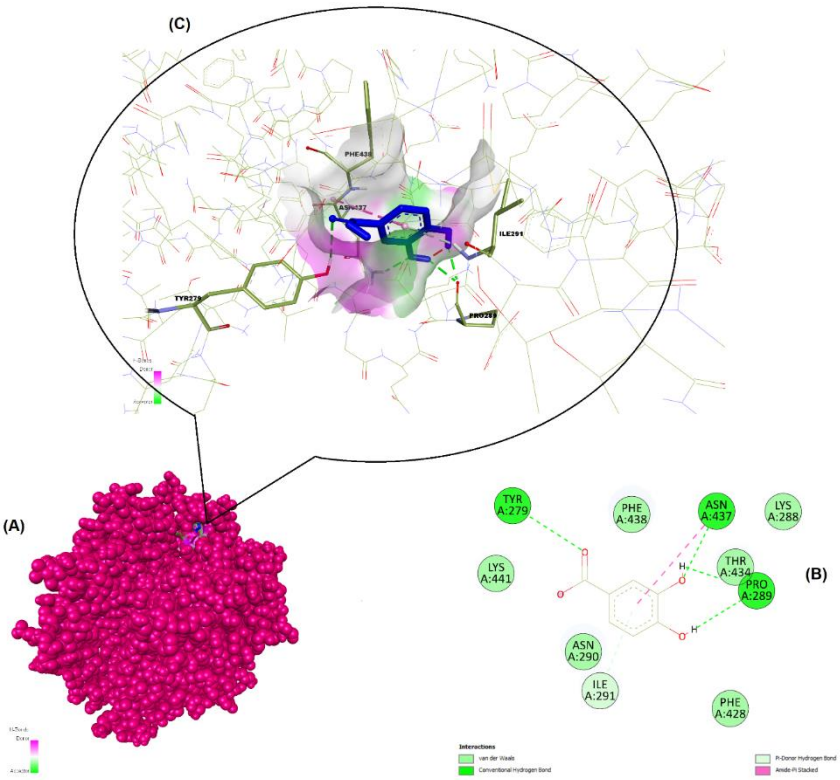


Figure 3. The binding pose profile of protocatechuic acid in the target protein ACE-2 (A) is depicted, showing the magenta-colored molecule receptor and the blue-colored molecule ligand. Two-dimensional (2D) (B) and three-dimensional (3D) (C) interaction analyses of ACE-2 protein with protocatechuic acid are presented.

Molecular docking is a crucial tool for exploring interactions between a target protein and a small molecule. Binding energy data (kcal/mol) allows us to examine and compare the binding affinity of different ligands/compounds with their respective target receptor molecules. Lower binding energy indicates a higher affinity of the ligand for the receptor. The ligand with the highest affinity can be selected as a potential drug for further studies. For this study, protocatechuic acid with a wide range of biological activities were used along with hydroxychloroquine (positive control), which demonstrated activity against SARS-CoV-2. The binding affinities of these ligands with the SARS-CoV-2 Spike Protein RBD and ACE-2 which

were used as receptors, were investigated. In a study conducted by Guler and his colleagues in 2021, docking analyses were performed with many flavonoids using the same receptors, and effective binding profiles were observed. Similarly, effective results were found in these docking analyses performed with protocatechuic acid, which was not included in that study (Guler et al., 2020).

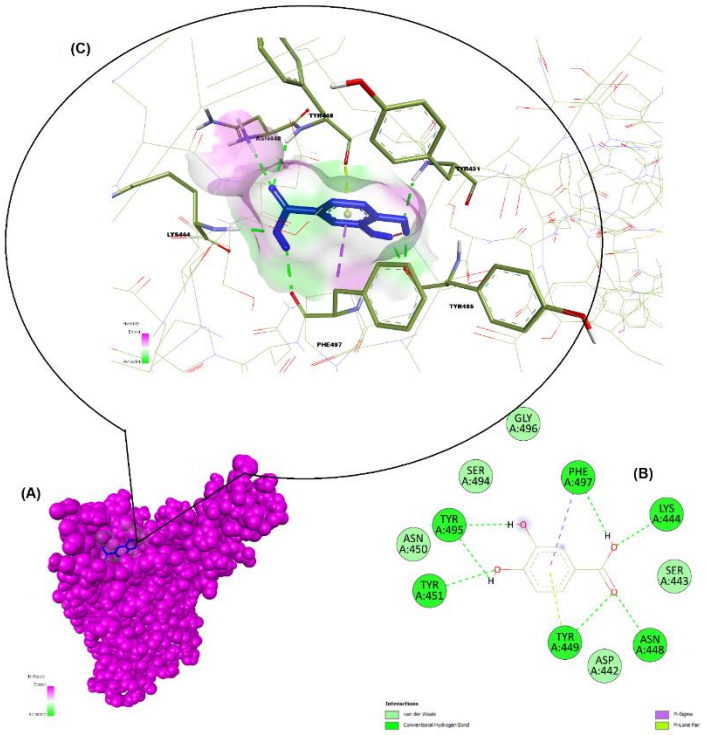


Figure 4. The binding pose profile of protocatechuic acid in the target protein SARS-CoV-2 Spike RBD (A) is depicted, showing the magenta-colored molecule receptor and the blue-colored molecule ligand. Two-dimensional (2D) (B) and three-dimensional (3D) (C) interaction analyses of SARS-CoV-2 Spike RBD with protocatechuic acid are presented.

Ligand protocatechuic acid and a reference molecule were individually docked to ACE-2 and SARS CoV-2 Spike RBD, respectively. After successful docking of all ligands used in these docking experiments, the results showed us that the protocatechuic acid performed significant interactions with the target receptors. The results indicate that, the ligand bound to the ACE-2 protein is effectively similar to the reference molecule. When the docking results with ACE-2 protein were examined, it was seen that protocatechuic acid has formed three conventional hydrogen bonds, one amide pi-stacked bond, one donor-donor bond and one pi-donor bond, three of these bonds had an atomic distance lower than 3 Å. The strongest bond of that interaction formed at position Pro289 with a length of 2.13 Å. When the docking results with Spike RBD were examined, it was seen that protocatechuic acid had better binding than the

reference molecule. Protocatechuic acid was found to be the molecule that bound strongly to the relevant receptor (-7.54 kcal/mol and 2.98 μM). It was observed that 8 conventional hydrogen bonds and 1 Pi-lone bond were formed in this docking and that conventional hydrogen bonds formed very effective bonds with a length of 1.88 Å at the Try495 position and 1.76 Å at the Lys444 position (Figure 3). The binding levels and details of the best interacting ligands are shown in detail in Table 4. For docking protocol validation, Spike receptor binding domain and its original native inhibitor (CR3022 Fab) were redocked and RMSD value was calculated. RMSD value of 1.94 Å between the docked conformation of the inhibitor and native conformation depicted the accuracy of the docking program.

Table 4. Estimated binding affinity (Kcal/mol), Ki values, and amino acids interacting at binding sites of docked ligands against ACE-2 and SARS-CoV-2 Spike receptor

| **Receptor Name / PDB ID** | **Ligand Name** | **Binding Energy (kcal/mol)** | **$K_i$ (μM)** | **H bonds** | **Interacted residues with ligand** |
|---|---|---|---|---|---|
| **Angiotensin-converting enzyme 2 (ACE-2) EC: 3.4.17.23 / 6M0J (Chain A) Res: 2.45 Å** | Protocatechuic acid | -5.25 | 141.22 | 3 | Ile291, Pro289, Asn437, Phe438 |
| | *Hydroxychloroquine | -7.90 | 1.61 | 4 | Arg393, Phe390, Leu391, Asn394, His378, His401, Asp350 |
| **SARS-CoV-2 Spike receptor binding domain / 6YLA (Chain A) Res: 2.42 Å** | Protocatechuic acid | -7.54 | 2.98 | 8 | Lys444, Asn448, Tyr449, Tyr495, Tyr451, Phe497 |
| | *Hydroxychloroquine | -6.32 | 23.35 | 7 | Leu517, Tyr396, Val382, Phe392, Thr430, Phe515 |

*reference molecule

## 4. CONCLUSION

The composition of propolis extracts depends on many factors, such as the flora of the region where the raw propolis is collected, the time of collection, and the extraction techniques. Therefore, it is not easy to standardize propolis extracts. In this study, propolis samples were collected from three different regions, and their effects on SARS-CoV-2 spike S1 protein and the ACE-2 receptor interaction were investigated. The propolis sample demonstrating the highest inhibition effect was identified as Kocaeli-1 propolis. Through this study, the phenolic content of Kocaeli-1 propolis was determined, and the effects of these phenolics on SARS-CoV-2 spike S1 protein and the ACE-2 receptor interaction were individually examined and docking studies were carried out to demonstrate the interactions of the molecules deemed effective with both molecules (ACE-2, Spike RBD) separately and interactively. It was observed that many molecules in propolis effectively bind to the ACE-2 protein. When comparing in silico results with in vitro findings, caffeic acid and protocatechuic acid were observed to have considerable binding affinities to both the SARS-CoV-2 spike S1 protein and the ACE-2 receptor.

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## DECLARATIONS

No conflict of interest or common interest has been declared by the authors.

## AUTHOR CONTRUBITIONS

The authors confirm contribution to the paper as follows: Study conception and design, data collection: Sabriye CANAKCI and Ali Osman BELDUZ**;** analysis and interpretation of results: Halil İbrahim GÜLER, Fulya AY**;** draft manuscript preparation: Fulya AY**.** All authors reviewed the results and approved the final version of the manuscript.

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