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**An investigation of ethanolic propolis extracts: Their potential inhibitor  
properties against ACE-II receptors for COVID-19 treatment by Molecular  
Docking Study**

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**Conflict of interest**

No conflict of interest is declared.

## **Abstract**

The angiotensin-converting enzyme (ACE)-related carboxypeptidase, ACE-II, is a type I integral membrane protein of 805 amino acids that contains one HEXXH-E zinc binding consensus sequence. ACE-II has been implicated in the regulation of heart function and also as a functional receptor for the coronavirus that causes the severe acute respiratory syndrome (SARS). In this study, the potential of some flavonoids present in propolis to bind to ACE II receptors was calculated *in silico*.

Binding constants of ten flavonoids, caffeic acid, caffeic acid phenethyl ester, chrysin, galangin, myricetin, rutin, hesperetin, pinocembrin, luteolin and quercetin were measured using the AutoDock 4.2 molecular docking program. And also, these binding constants were compared to reference ligand of MLN-4760.

The results are shown that rutin has the best inhibition potentials among the studied molecules with high binding energy -8,97 kcal/mol and Ki 0,261  $\mu$ M, and it is followed by myricetin, caffeic acid phenethyl ester, hesperetin and pinocembrin. However, the reference molecule has binding energy of -7,28 kcal/mol and 4,65  $\mu$ M. In conclusion, the high potential of flavonoids in ethanolic propolis extracts to bind to ACE II receptors indicates that this natural bee product has high potential for Covid- 19 treatment, but this needs to be supported by experimental studies.

**Keywords:** Coronavirus, Covid-19, propolis, flavonoids, ACE-II, molecular docking

## **Introduction**

Propolis is a natural mixture that honey bees collect from nature in order to protect their hives. Honey bees use propolis for insulating hives, mummification of dead insects and bees and as an antibacterial, antiviral, antioxidant and anti-inflammatory agent for many biological activities. Crude propolis is a highly viscous, slightly soluble mixture in water and best dissolved in 60-80% ethanol. Propolis has been an indispensable component of apitherapy for centuries and has recently been used as a food additive, or supplementary, under the name of traditional and complementary medicine [1, 2].

Its composition varies according to the flora of the region where it is collected, but the majority of active ingredients of propolis comprise the family of polyphenols. Phenolic acids, flavonoids (flavanones, flavones, flavonols etc.), stilbenes, tannins are the active polyphenols

of propolis [1, 3]. Propolis are not consumed as raw, but their ethanolic and aqueous extracts are widely consumed in different formulations.

It has been reported that polyphenolic agents such as gallic acid, caffeic acid, protocatechuic acid, chrysin, quercetin, rutin, galangin, kaempferol, hesperetin, pinocembrin, pinobanksin, apigenin, luteolin, daidzein, caffeic acid phenyl ester (CAPE) are the most active polyphenols of propolis and these secondary metabolites vary depending on the propolis source [1, 4, 5].

Studies show that propolis extracts have high immunomodulatory effect and inhibition potential for some clinically important enzymes, such as urease, xanthine oxidase (XO), acetylcholinesterase (AChE),  $\alpha$ -amylase,  $\alpha$ -glucosidase [6, 7]. In addition, *in vivo* and *in vitro* studies show that flavonoids, one of the active ingredients of propolis, have high potential for Angiotensin-Converting Enzyme (ACE) Inhibition [8, 9, 10].

The newly discovered SARS-CoV-2 was characterized as a beta-coronavirus and recognized as the seventh discrete coronavirus species capable of causing human disease. The disease caused by the virus is officially named Coronavirus Disease 2019 (Covid-19) by World Health Organization (WHO). The emerged global epidemic spread rapidly with 2.246.291 confirmed cases and 152.707 deaths across 213 countries, areas and territories (COVID-19 situation Report WHO, 20 April 2020). Subsequent studies have shown that SARS-CoV-2 has been suggested to recognize human ACE II more strongly than SARS-CoV, thereby increasing the ability to be transmitted from person to person [11]. Therefore, ACE II enzyme inhibition is important for treatments against these virus infections caused by SARS-CoV-2.

The aim of this study was to calculate the inhibition constants of some flavonoids, one of the active ingredients of Anatolian propolis, to the ACE II enzyme by molecular modeling as a positive control (S, S) -2- {1-Carboxy-2- [3- (3,5-Dichloro-Benzyl) -3h-Imidazole-4-Yl] -Ethylamino}-4-Methyl-Pentanoic Acid (MLN-4760). We analyzed therapeutic potential compounds of Turkish propolis extracts tested against ACE-II in experimental studies with *in silico* methods.

Up to now, there are very limited studies about Covid-19 and most researchers focused primarily on clinical cases. However, to the authors' knowledge, no study has been made on inhibition of ACE-II known to be associated with Covid-19. Therefore, the paper will encourage further researches about Covid-19 and candidate drug compounds.

## **Materials and Methods**

### **Materials**

Raw propolis samples obtained experienced beekeepers in 2018 from Black Sea Region, Turkey.

### **Chemicals**

All phenolic standard for HPLC analyses of gallic acid, protocatechuic acid, *p*-OH benzoic acid, catechin, caffeic acid, syringic acid, epicatechin, *p*-coumaric acid, ferulic acid, rutin, myricetin, resveratrol, daidzein, luteolin, *t*-cinnamic acid, hesperetin, chrysin, pinocembrin, caffeic acid phenethyl ester (CAPE) were purchased from Sigma Chemical Co.(St Louis, MO, USA). All solvent for using mobile phases were analytical grade.

### **Preparation of propolis extracts**

The raw propolis samples were frozen at -20 °C and then grinded to powder. The following method was used to prepare the ethanolic propolis extract: 10 g powdered crude propolis was placed with 100 mL 70% ethanol in a glass flask and stirred with shaker (Heidolph Promax 2020, Schwabach, Germany) at room temperature for 24 h and then filtered with Whatman paper.

### **Determination of phenolic profiles**

In this study, nineteen phenolic standards were used to high-performance liquid chromatography (HPLC) (Elite LaChrom Hitachi, Japan) with a UV detector. Separation was performed on a column with a reverse phase C18 column (150 mmx4.6 mm, 5µm; Fortis), in gradient solvent systems A (2% acetic acid in water) and solvent B (70:30, acetonitrile/water), which was sonicated before stirring and continuously degassed by the built-in HPLC system [12, 13]. The flow rate was kept constant at 1 mL/min using gradient programming, starting the flow of the mobile phase as B (5%) to 3 minutes, gradually increasing (up to 15, 20, 25, 40 and 80% at 8, 10, 18, 25 and 35 minutes, respectively) and decreasing to 5% at 40 minutes, before being left for 10 minutes to equilibrate in the column. The standard phenolic substances chromatogram is given in Figure 1.

### **Molecular Docking**

In the study, some flavonoids detected in the ethanolic propolis extracts and they used as ligand (see in Supplementary File) for ACE II receptors. The crystal structure of a ACE II protein was downloaded from protein data bank web site (<http://www.rcsb.org/pdb>) (PDB ID:

1R4L: Resolution 3.00 Å). This crystal structure contains the inhibitory bound state of the extracellular metalloproteinase domain of ACE II with MLN-4760 compound. Small compounds of flavonoids used in docking studies were obtained from PubChem as SDF form and were drawn in the Hyperchem software [14] then subjected to conformational search with geometric optimization. Possible docking modes between compounds and the ACE II enzyme were studied using the Autodock 4.2 [15] and Lamarckian genetic algorithm was employed for docking simulations. The selected cavity is the binding site of reference inhibitor MLN-4760 [ ((S,S)-2-{1-Carboxy-2-[3-(3,5-dichloro-benzyl)-3H-imidazol-4-yl]-ethylamino}-4-methyl-pentanoic acid)]. A grid box dimensions of 52, 34, and 47 points in x, y, and z directions was set with a grid spacing of 0.375 Å. The program was run for a total number of 100 Genetic algorithm runs. The default settings were applied for all other parameters. Results of the molecular docking described the affinity represented by docking score and binding interaction of each ligand on the interested protein target. The visualization of results was performed with the help of the BIOVIA Discovery Studio 2018 [16].

## **Results and Discussion**

### **Phenolic composition of propolis extract**

In this study, a standard HPLC-UV chromatogram prepared with nineteen phenolic standards including some phenolic acids and flavonoids is given in Fig 1. The analysis data of the ethanolic propolis extract carried out according to this chromatogram are summarized in Table 1. Although the hydroxybenzoic acids and catechin derivatives of the propolis sample were found below determination limits, it was found to be rich in hydroxycinnamic acids and flavanoids. Among the hydroxycinnamic acid derivatives, the caffeic acid phenyl ester is the highest amount of phenolic component in the sample and followed it caffeic acid and cinnamic acid. Ferulic acid could not be detected in the sample. Among the flavonoids subclass of flavonoids, the highest amount of myricetin was detected and rutin followed it.

Among these three flavanons studied, chrysin is the most abundant compounds pinosebrin and hesperetin followed. A smaller amount of flavone derivative of luteolin, was detected, while daidzein is not detected. Of all the studied compounds, chrysin and pinosembrin were detected as major flavonoids in the propolis sample.

Although composition of propolis varies according to the flora of the region where it is produced, these flavonoids were also reported in propolis samples of different countries [17, 18, 19]. There are many scientific studies showing that propolis, a natural bee product, is a very rich mixture of flavonoids and is an important agent of apitherapy. Polyphenolic profile

of propolis varies according to the flora of the region where it is collected, caffeic acid, CAPE, rutin, quercetin, polyphenols such as myricetin, kampherol, hesperetin, galangin are the active substances of Anatolian propolis [3, 4, 5, 20, 21]. Barbarić et al. (2011) studied chemical composition of the ethanolic propolis extracts and determined ferulic acid, p-coumaric acid, caffeic acid, tectochrysin, galangin, pinocembrin, chrysin, apigenin, kaempferol, quercetin as phenolic compound in Croatia, Bosnia and Hercegovina and Macedonia propolis [22]. Major compounds of red propolis samples from Brasilia were found as luteolin (1.75 mg/g), naringenin (0.96 mg/g), kaempferol (0.59 mg/g), pinocembrin (0.41 mg/g) and biochanin A (0.39 mg/g) [23]. There are some differences between the findings because the chemical composition of propolis varies according to the geographical region, climate, environmental conditions and collection seasons [23, 24, 25]. The findings show that propolis are a good source of phenolic substances. The literature states that propolis samples from different geographical origins have a good antioxidant antimicrobial, antifungal and antiviral (Avian influenza virus) activity [26, 27, 28, 29, 30].

### **Binding affinity analysis for proteins and ligands with molecular docking**

We focus here on the Anatolian propolis compounds used by people to treat infections against ACE II with molecular docking methods. For this purpose, we made docking analysis with the compounds and found that quercetin, rutin, myricetin and hesperetin have a better affinity against ACE II enzyme than natural inhibitor MLN-4760 with low  $\mu\text{M}$   $K_i$  values among the evaluated compounds (Table 2).

Furthermore, these compounds interacted with *Arg273*, *Thr371*, *His345*, *Pro346*, *Tyr515*, *Glu402* and *Glu375* in ACE II binding site. Especially, our *in silico* study showed that, rutin has the best binding affinity to the ACE II enzyme (Binding energy: -8.98 kcal/mol,  $K_i$  0.261  $\mu\text{M}$ ). This compound was observed to bind to the residues *Asn149*, *His345*, *Asp269*, *Glu375*, *Glu406*, *Thr371*, *Tyr127* and *Asp368* of ACE II protein with the stronger hydrogen bond (Figure 2). It can be suggested that these residues can contribute to the enhancement of ligand affinity for ACE II enzyme. In addition, this compound has the pi-cation interaction with *Arg273*, pi-pi T shape interaction with *His374*, alkyl interaction with *Cys344* and pi-alkyl interaction with *Tyr 127* residues (Figure 2).

Therefore, in this study, *in silico* effects of Anatolian propolis on ACE II enzyme inhibition was investigated with the ten flavonoids as major substances. The results of this study showed that quercetin, rutin, myricetin and hesperetin compounds effectively inhibit the ACE II enzyme. These compounds can be clinically tested and used for the treatment disease

role of ACE II. Furthermore, *Arg273, Thr371, His345, Pro346, Tyr515, Glu402 and Glu375* are potential inhibitor targeting sites for the ACE II enzyme. Based on this information, we propose guidelines to develop novel and specific inhibitors that target ACE II enzyme.

Guerrero et al. (2012) experimentally demonstrated that some flavonoids have a relatively high inhibition potential for ACE-I [31]. With the molecular docking studies, we have shown that some of these flavonoids inhibit ACE-II. ACE-I and ACE-II enzymes are metalloproteases, both of which contain similar zinc fingers (HEXXH) in their active sites. Molecular docking studies indicated that there are bond interactions between rutin and zinc finger residues of ACE II. Because of similar active sites of ACE I and II, rutin may functionally bind both ACE I and II similar way.

It is revealed that Covid-19 binds to human angiotensin-converting enzyme 2 (ACE2) to enter the host cells. Rutin may compete with Covid-19 for ACE II and may prevent or delay the entry of Covid-19 into the cell.

In recent years, flavonoids have gained a great amount of interest with regards to their potential for cardiovascular protection. In fact, many epidemiological studies associate an increased consumption of foods and beverages rich in flavonoids with a reduced risk of CVD death [32, 33, 34]. Additionally, several of these flavonoids or their derivatives (i.e., diosmin, rutin and quercetin) are widely used as pharmaceutical agents for their vasoprotective properties (i.e., Daflon 500, containing flavonoid derivatives hesperedin and diosmin) [35]. Therefore, rutin and other flavonoids used in this study can be used for prophylactic purposes as ACE II inhibitors and competitor [36, 37].

In conclusion, *in silico* study is shown that the high binding constants for the ACE II receptors of flavanones in the ethanolic propolis extract make it a good competitive inhibitor and protective natural agents for the treatment of Covid-19. However, this study should be supported with further *in vivo* studies.

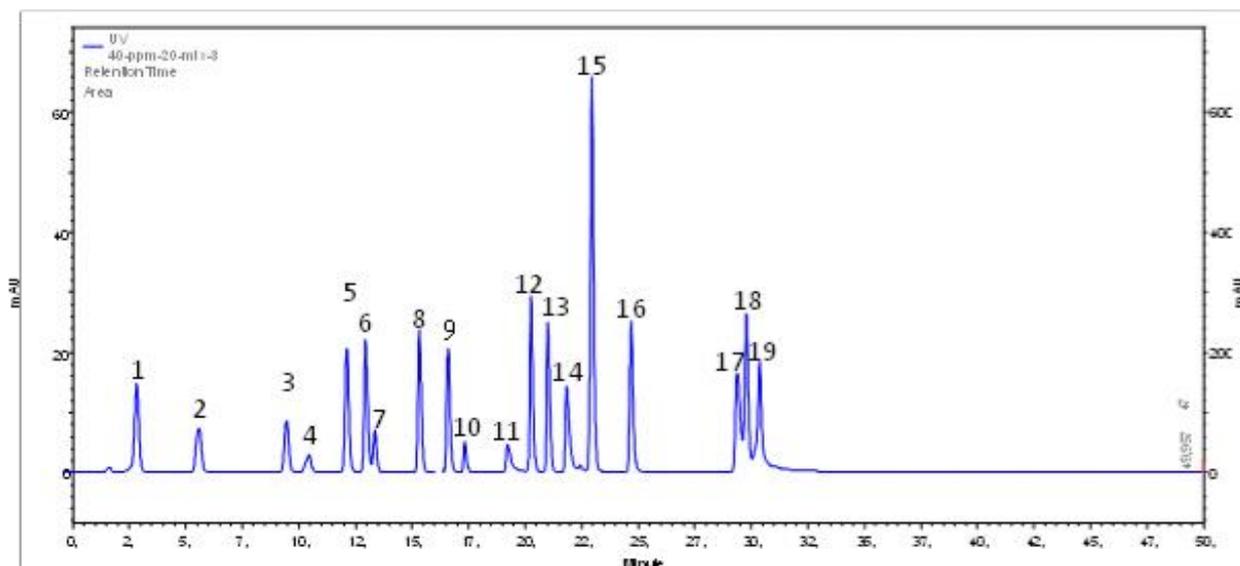
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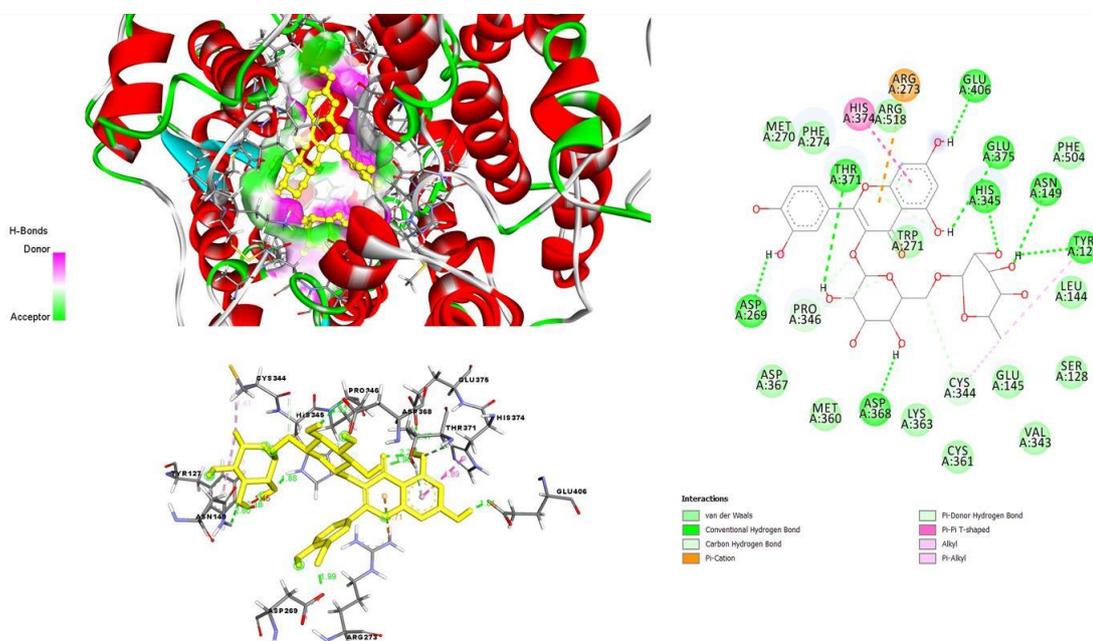
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**Figure 1.** Chromatogram of nineteen phenolic compounds of HPLC-UV; (1): Gallic acid, (2): Protocatechuic acid, (3): p-OH benzoic acid, (4): catechin, (5): caffeic acid, (6): syringic acid, (7): epicatechin, (8): p-coumaric acid, (9): ferulic acid, (10): rutin, (11): myricetin, (12): resveratrol, (13).daidzein, (14); luteolin, (15): t-cinnamic acid, (16): hesperetin, (17): chrysin, (18):pinocembrin, (19): Caffeic acid phenethyl ester (CAPE).



**Figure 2.** The two-dimension (2D) and three-dimension (3D) interaction analysis of ACE II with rutin

**Table 1.** Phenolic composition of propolis

| <b>Group name</b>            | <b>Compound name</b>    | <b>mg/100g</b> |
|------------------------------|-------------------------|----------------|
| <i>Hydroxybenzoic acids</i>  | Gallic acid             | n.d.           |
|                              | Protocateuic acid       | n.d.           |
|                              | p-OH Benzoic acid       | n.d.           |
|                              | Syringic acid           | n.d.           |
| <i>Catechin</i>              | Catechin                | n.d.           |
|                              | Epicatechin             | n.d.           |
| <i>Hydroxycinnamic acids</i> | Caffeic acid            | 254.501        |
|                              | <i>p</i> -Coumaric acid | 63.871         |
|                              | Ferulic acid            | n.d.           |
|                              | <i>t</i> -Cinnamic acid | 145.455        |
|                              | CAPE                    | 541.213        |
| <i>Flavanons</i>             | Rutin                   | 770.970        |
|                              | Myricetin               | 1567.750       |
| <i>Flavones</i>              | Hesperetin              | 258.010        |
|                              | Chrysin                 | 4678.423       |
|                              | Pinocembrin             | 1467.260       |
| <i>Isoflavons</i>            | Luteolin                | 684.752        |
| <i>Stilbands and Lignans</i> | Daidzein                | n.d            |
|                              | Resveratrol             | 2.372          |

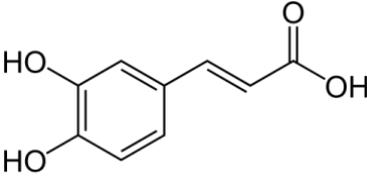
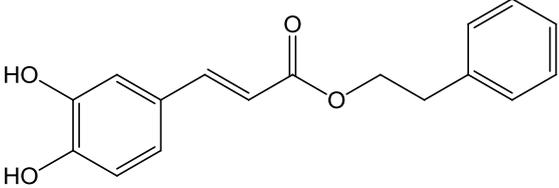
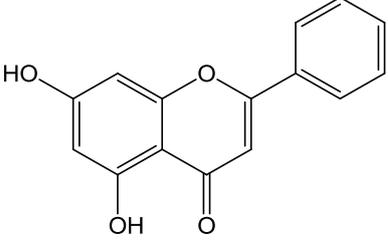
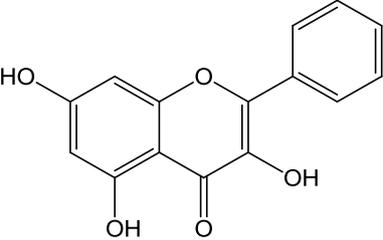
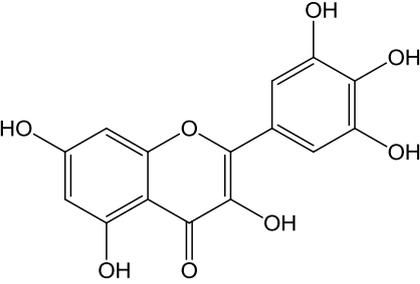
n.d: not detected

**Table 2:** Summary of reference and flavonoids compounds against ACE II with binding energy, Ki and interacted residues in the ACE II binding site.

| Ligand Name   | Binding Energy (kcal/mol) | Ki (uM) | ACE II residues interacting with ligands   |
|---|---------------------------|---------|--|
| MLN-4760 ((S,S)-2-{1-Carboxy-2-[3-(3,5-Dichloro-Benzyl)-3h-Imidazol-4-Yl]-Ethylamino}-4-Methyl-Pentanoic Acid)*                                     | -7.28                     | 4.65    | Tyr127, Arg273, Phe274, Trp271, Arg273, Phe274, His345, Pro346, Cys361, Thr371, His374, Glu375, His378, Glu406, Phe504, His505, Tyr515, Arg518   |
| Caffeic acid (3,4-dihydroxycinnamic acid)   | -5.53                     | 89.08   | Arg273, His345, Pro346, Thr347, Ala348, His374, Glu375, His378, Phe504, His505, Tyr515   |
| Caffeic acid phenethyl ester (Caffeic acid 2-phenylethyl ester; $\beta$ -Phenylethyl caffeate) (2-Phenylethyl (2E)-3-(3,4-dihydroxyphenyl)acrylate) | -7.76                     | 2.04    | Tyr127, Ser128, Leu144, Glu145, Asn149, Trp271, Val343, Cys344, His345, Pro346, Met360, Cys361, Thr362, Lys363, Asp368, Phe504   |
| Chrysin (5,7-Dihydroxyflavone)  | -7.08                     | 6.41    | Tyr127, Ser128, Glu145, Asn149, Cys344, His345, Pro346, Met360, Cys361, Thr362, Lys363, Asp368, Phe504   |
| Galangin (3,5,7-Trihydroxyflavone)  | -7.18                     | 5.41    | Arg273, Phe274, His345, Pro346, Thr347, Ala348, Thr371, His374, Glu375, His378, Glu406, Phe504, His505, Tyr515, Arg518   |
| Myricetin (3,3',4',5,5',7-Hexahydroxyflavone)   | -7.70                     | 2.28    | Arg273, Phe274, His345, Pro346, Thr347, Ala348, Thr371, His374, Glu375, His378, Glu406, Phe504, His505, Tyr515, Arg518,  |
| Rutin (Quercetin-3-rutinoside hydrate)  | -8.98                     | 0.261   | Tyr127, Ser128, Leu144, Glu145, Asn149, Asp269, Met270, Trp271, Arg273, Phe274, Val343, Cys344, His345, Pro346, Met360, Cys361, Lys363, Asp367, Asp368, Thr371, His374, Glu375, Glu406, Phe504, Arg518 |
| Hesperetin (3',5,7-Trihydroxy-4'-methoxyflavanone)  | -7.40                     | 3.79    | Arg273, His345, Pro346, Thr347, Ala348, Trp349, Thr371, His374, Glu375, His378, Glu406, Phe504, His505, Tyr510, Tyr515, Arg518   |
| Pinocembrin (5,7-Dihydroxy-2-phenyl-2,3-dihydro-4H-chromen-4-one)   | -7.46                     | 3.38    | Tyr127, Ser128, Leu144, Glu145, Asn149, Trp271, Val343, Cys344, His345, Pro346, Met360, Cys361, Thr362, Lys363, Asp368   |
| Luteolin (2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4-chromenone)   | -6.93                     | 8.36    | Arg273, Phe274, His345, Pro346, Thr347, Ala348, Trp349, His374, Glu375, His378, Asp382, Glu402, Phe504, His505, Tyr510, Tyr515, Arg518   |
| Quercetin   | -7.62                     | 2.62    | Arg273, Phe274, His345, Pro346, Thr347, Ala348, Thr371, Glu375, His374, His378, Glu406, Phe504, His505, Tyr515, Arg518   |

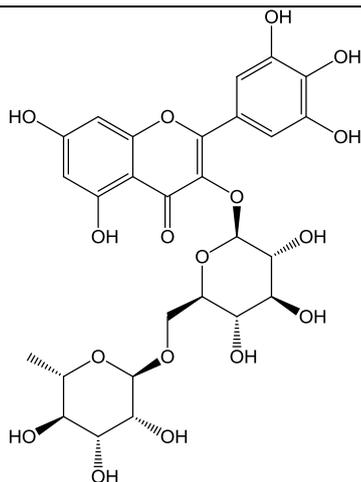
\*Reference compound

Supplementary Table: Chemical compounds used for the molecular docking screening

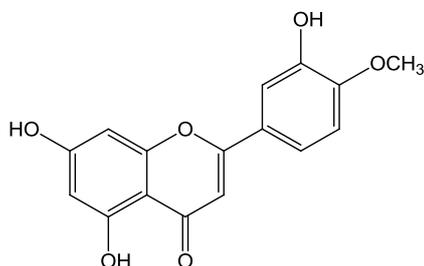
| Chemical compound   | 2D-Structure   |
|---|--|
| Caffeic acid (3,4-dihydroxycinnamic acid)   |    |
| Caffeic acid phenethyl ester (Caffeic acid 2-phenylethyl ester; $\beta$ -Phenylethyl caffeate) (2-Phenylethyl (2E)-3-(3,4-dihydroxyphenyl)acrylate) |    |
| Chrysin (5,7-Dihydroxyflavone)  |   |
| Galangin (3,5,7-Trihydroxyflavone)  |  |
| Myricetin (3,3',4',5,5',7-Hexahydroxyflavone)   |  |

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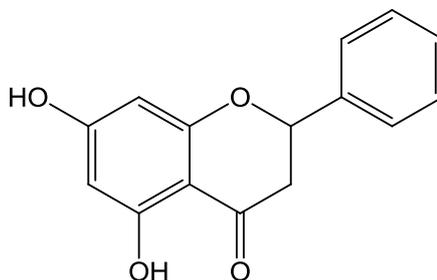
Rutin (Quercetin-3-rutinoside hydrate)



Hesperetin (3',5,7-Trihydroxy-4'-methoxyflavanone)



Pinocembrin (5,7-Dihydroxy-2-phenyl-2,3-dihydro-4H-chromen-4-one)



Luteolin (2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4-chromenone)

