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Chemical composition, antimicrobial activities, and molecular docking studies of Turkish propolis ethanol extract

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Abstract: The purpose of the present study was to investigate the antimicrobial effect of propolis ethanol extract collected from the Tarsus district of Mersin province, Kilis province, Yayladagi district of Hatay province in southern Türkiye and Sarkoy district of Tekirdag province of northwestern Türkiye against *Escherichia coli* (ATCC 25922), *Helicobacter pylori* (ATCC 43504), *Pseudomonas aeruginosa* (ATCC 27853), and *Staphylococcus aureus* (ATCC 29213). Their chemical constituents were detected via liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). They were used in a molecular docking approach to search the interactions between the propolis compounds. A total of 24 phenolic compounds were detected in all samples. 3–4 dimethoxycinnamic acid, caffeic acid and genistein were indicated to be the predominant phenolic compounds in propolis extracts by LC-MS/MS, while rutin was found in the lowest concentration. Phenolic compounds were detected in a high concentration of the propolis samples collected from the Tarsus district of Mersin province. The broth microdilution method determined minimum inhibition concentration (MIC) values. MIC values ranged from 0.02 to 14 mg·mL^{-1}. *E. coli* and *S. aureus* examined were as susceptible to the propolis extracts except for Mersin and Tekirdag propolis samples. The propolis sample collected from the Tarsus district of Mersin province presented the highest antibacterial activity on *P. aeruginosa* with MIC values of 1 mg·mL^{-1}. Active substances in propolis were docked to the relevant target proteins (5LMM, 4NX9, 5YHG, and 5FXT) representing *E. coli* (ATCC 25922), *H. pylori* (ATCC 43504), *P. aeruginosa* (ATCC 27853), and *S. aureus* (ATCC 29213), and with the help of molecular simulation. With this study, we indicated that the ethanol extract of propolis had a stronger antibacterial activity on *S. aureus* isolates than that of *E. coli*, *H. pylori*, and *P. aeruginosa.* Although each component of propolis contributed to the antibacterial activity, the contribution of the vitexin component to the antibacterial activity was found to be quite significant.

Keywords: antimicrobial effect; minimum inhibition concentration (MIC); phenolic compounds; vitexin; liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS)

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Propolis is a bee glue and an important antimicrobial bee product that acts against Gram-positive and Gram-negative bacteria, and its activity is related to chemical composition. It is different in all countries (Przybyłek and Karpiński 2019). More than 300 chemical compounds of propolis have been determined (Przybyłek and Karpiński 2019). Polyphenols such as phenolic acids and flavonoids and terpenoids are considered to be the most active (Pimenta et al. 2015). The flavonoid group contains apigenin, chrysin, galangin, kaempferol, pinocembrin, pinostrobin, quercetin, tectochrysin, and others. Other important groups of chemical constituents in propolis are aromatic acids such as benzoic, caffeic, cinnamic, ferulicsalicylic and p-cumaric acids (Kędzia and Hołderna-Kędzia 2017). Determining the antimicrobial activity of propolis may be useful in treating and preventing diseases (Babiker et al. 2020).

To date, several methods, such as simple agar diffusion or dissolution tests, have been employed to assess the antimicrobial effect of propolis by in vitro test systems. Using the broth microdilution method, the latter test detects the minimum inhibitory concentration (MIC) or the minimum bactericidal concentration (MBC). Agar diffusion tests only indicated the inhibition of bacterial growth (Grecka et al. 2019). These tests are widely used to detect the agents' potential and categorise them in connection with alternative chemical materials (Rajini et al. 2017; Grecka et al. 2019). Molecular docking studies associate the biological activities of chemical species with structure-based properties (Aydın et al. 2021; Khalid et al. 2021). Recently, it has become one of the areas required in studies exhibiting cellular activity. The target representing the biological macromolecule can be determined from the protein database. The activity of the studied chemical species can interact with target proteins through a simulation. In this way, the interactions between the protein is representing the cell and the chemical species examined can be examined in terms of energy and binding mode at the molecular level (Güzel et al. 2021).

The study aimed to determine the constituents of polyphenols (phenolic acids and flavonoids) using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) in the propolis samples collected from the different provinces of Türkiye and to detect MIC using the broth microdilution method from ethanolic extract of propolis against four pathogens such as *Pseudomonas aeruginosa* (*P. aeruginosa*, American Type Culture Collection – ATCC 27853), *Escherichia coli* (*E. coli*, ATCC 25922), *Helicobacter pylori* (*H. pylori*, ATCC 43504), and *Staphylococcus aureus (S. aureus*, ATCC 29213). In addition, the active substances in propolis were docked to the relevant target proteins representing *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *S. aureus* (ATCC 29213), and *H. pylori* (ATCC 43504) with the help of molecular simulation.

MATERIAL AND METHODS

Collection and extraction of propolis. The trap propolis samples were collected in the summer from the Tarsus district of Mersin province, Kilis province, Yayladagi district of Hatay province in southern Türkiye and Sarkoy district of Tekirdag province of northwestern Türkiye in 2010, 2012, 2013, and 2016, respectively. The frozen crude propolis specimens were storaged at –20 °C (Laboratory Grinder Set – ISOLAB).

Ethanolic propolis extracts were obtained according to the protocol described by Devequi-Nunes et al. (2018). *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *S. aureus* (ATCC 29213), and *H. pylori* (ATCC 43504) (kindly ensured by Professor Francis Megraud from Pellegrin Hospital, Bordeaux, France) strains were used as test bacteria in this study. The broth microdilution method determined the propolis extract's MIC values, which is the reference method for antimicrobial susceptibility tests according to the Clinical and Laboratory Standards Institute (CLSI) protocol (CLSI 2009). Briefly, the propolis extract was serially diluted twofold in a 96-well microplate containing dilutions of the antimicrobial agent. To each tube, 5×10^5 CFU·mL^{-1} (colony-forming units per milliliter) from the microbial suspension of each bacteria equivalent to a 0.5 McFarland density standard was added. Turbidity was read and recorded after 18–24 h of incubation at 37 °C. The last dilution in that microbial opacity was visible was recorded as the minimum microbial concentration (Zeighami et al. 2015). The lowest antimicrobial agent concentration at which the growth of a microorganism was visibly inhibited is detected as the 'MIC' (Babiker et al. 2020). Colistin for *E. coli* and *P. aeruginosa* and vancomycin for *S. aureus,* and dimethyl sulfoxide (DMSO) were used as positive controls, and the 80% ethanol solution as a negative control in all steps of the experiment.

Two grams of propolis was weighed and dissolved in 15 mL of 80% ethanol and homogenised propolis and then incubated at 70 °C and 710 rpm in a Shaker incubator (Park et al. 2002). After then, the extract was centrifuged at 8 800 rpm and 5 °C for 10 min, the su-

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pernatant was placed in test tubes and 10 mL of 80% ethanol was added and centrifuged (Machado et al. 2016). Sterilisation was performed by passing through a 0.22 µm microfilter. All the extracts were stored at a 5 °C to avoid degradation. Colistin for *E. coli* (MIC values, 1 mg·mL^{-1}) and *P. aeruginosa* (MIC values, 2 mg·mL^{-1}) and vancomycin for *S. aureus* (MIC values, 1 mg·mL^{-1}) and DMSO were used as positive controls and the 80% ethanol solution as a negative control. All experiments were carried out in duplicate.

LC-MS/MS analysis. The LC-MS/MS method was carried out according to the method described by Runyoro et al. (2017). AB Sciex 3200 triple quadrupole mass spectrometer (QTRAP) (Applied Biosystems/MDS Sciex, USA) was used as a mass spectrophotometry detector. Ionisation was made in positive and negative ion modes by the electrospray ionisation (ESI) module (LC-20 AD UFLC XR; Shimadzu Corporation, Japan), controlled by Analyst 1.6.1 software. The scan type was set to multiple reaction monitoring (MRM) modes. Ion spray voltage (IS) was set to 4 500 V, curtain gas (CUR) 30 psi, Ion Source gas 1 (60 psi), and Ion Source gas 2 (60 psi). The temperature of the TurboIonSprey module was fixed at 500 °C. Analyte-dependent parameters, working standard solution containing 0.1 mg·kg^{-1} of each standard substance was used for declustering potential (DP), collision energy (CE), collision cell entrance potential (CEP), and collision cell exit potential (CXP) (Runyoro et al. 2017).

Calculation method. Geometry optimisation of the investigated compounds was performed again with Merck Molecular Force Field 94 (MMFF94) method. The load calculation method was selected as Gasteiger. pH 7.0 was preferred in all calculations. In the placement calculations, grid maps 90 × 90 × 90 Â (x, y, and z), Lamarckian genetic algorithm (LGA), and Solis and Wets local search method was used (Güzel et al. 2021; Çakmak et al. 2022). At the time of insertion, the population size was set to 150. A translation step of 0.2 Å and 5 Å quaternion and torsion steps were done while searching for the appropriate region of the target protein of the molecules investigated (Nair et al. 2023).

Molecular docking. In this study, the main active ingredients in propolis were docked to target proteins representing bacterial species *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *S. aureus* (ATCC 29213), and *H. pylori* (ATCC 43504). Target proteins representing bacterial strains are from the protein data bank (PDB) ID: 5LMM (*E. coli* nife-hydrogenase hydrogen binding, oxidoreductase), 4NX9 (*P. aeruginosa* flagellin protein), 5YHG (*S. aureus* membrane protein), and 5FXT (crystal structure of *H. pylori* beta clamp in complex with carprofen), respectively. The determined target proteins and 3'4 dihydroxybenzoic acid, chrysin, naringenin, protocatechuic acid, pyrocatechol, rutin, vitexin, and vanillic acid ligands interacted with the DockingServer program. The chemical species that plays an active role in propolis components was investigated.

RESULTS AND DISCUSSION

The major phenolic compositions of propolis samples were detected using LC-MS/MS, and the values of each composition are shown in Table 1. Twenty four phenolic compounds were determined in all samples.

The highest flavonoids were found in propolis samples obtained from the Tarsus district of Mersin province. Four propolis samples from different regional origins were determined for their antibacterial activity against *E. coli*, *H. pylori*, *P. aeruginosa*, and *S. aureus*. The MIC results for all tested bacterial species are indicated in Table 2. As shown in Table 2, the MIC value of Yayladagi propolis samples was determined as 3 mg·mL^{-1} for *E. coli*, 2 mg·mL^{-1} for *H. pylori*, 0.9 mg·mL^{-1} for *P. aeruginosa*, and 0.05 mg·mL^{-1} for *S. aureus*.

It was determined that the propolis samples of Kilis province and Yayladagi district of Hatay province had the lowest MIC values and highest antibacterial activity against *S. aureus*. The MIC value of Tarsus propolis was found as 7 mg·mL^{-1} for *E. coli*, *H. pylori*, and *S. aureus*, and 1 mg·mL^{-1} for *P. aeruginosa*. Accordingly, the Tarsus propolis sample presented its highest effectiveness on *P. aeruginosa*. The MIC value of Sarkoy propolis was 7 mg·mL^{-1} for *E. coli*, and 14 mg·mL^{-1} for *H. pylori*, *P. aeruginosa*, and *S. aureus*. The propolis samples from Kilis province and Yayladagi district of Hatay province had a stronger inhibitory effect against *S. aureus* than against *E. coli*, *H. pylori*, and *P. aeroginosa*.

The docking results of the respective target proteins and active ingredients are listed in Table 3. When Table 3 is examined, it is noteworthy that the antibacterial activity of the studied ligands is high. In this case, ligands can be candidate molecules that can be used as antibacterial agents. The estimated binding energies between the studied ligands and the relevant target proteins gave different results according to the structure-activity relationship. These energies are derivatives of the interaction energies between the ligand

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Table 1. The phenolic compounds of propolis samples via LC-MS/MS method

Phenolic compound	Concentration of propolis (ppm)			
	Yayladagi district of Hatay province	Kilis province	Tarsus district of Mersin province	Sarkoy district of Tekirdag province
3-4 dimethoxycinnamic acid	250	294	620	277
Apigenin	145	72	78	103
Carvacrol	192	84	75	111
Galangin	122	74	84	98
t-cinnamic acid	52	102	113	40
Kaemferol	25	20	26	22
Alfa pinen	22	17	19	8
CAPE	103	100	123	151
Formononetin	378	169	145	232
Genistein	1 030	1 370	2 430	896
Genkwanin	17	5	6	13
Hesperidin	0.4	1.5	1.1	1.2
Hyperoside	1	3	2	2
Rac Naringenin	24	11	8	18
Naringenin	35	31	16	32
Quercetin	43	56	44	55
Rutin	0,8	3	2	2
Luteolin	26	12	17	28
3 hydroxy 4 methoxy cinnamic acid	38	164	91	27
p-coumaric acid	72	133	133	74
Cafeic acid	260	293	396	244
t-4 hydroxy 3 methoxy cinnamic acid	25	74	55	20
4 Hydroxy benzoic acid	25	19	28	8
Protocatechuic acid	46	28	30	10

LC-MS/MS – liquid chromatography-mass spectrometry/mass spectrometry

and the target protein, and the inhibition efficiency increases as the negative interaction energy increases. According to this approach, the propolis component with high inhibitory activity against each bacterial species is generally a vitexin ligand (Merugu et al. 2016). In addition, the interaction types of ligands with calculated antibacterial activity with target proteins are presented in Tables 4 and 5.

The secondary chemical interactions of the investigated components with the amino acid residues of the target proteins in Tables 4 and 5 are given in detail. Components interact differently with different target

Table 2. Minimum inhibition concentration (MIC, mg·mL^{-1}) results for all tested bacterial species

Propolis samples	*E. coli*	*H. pylori*	*P. aeruginosa*	*S. aureus*
Yayladagi-Hatay	3	2	0.9	0.05
Kilis	1	1	0.9	0.02
Tarsus-Mersin	7	7	1	7
Sarkoy-Tekirdag	7	14	14	14

E. coli – Escherichia coli, H. pylori – Helicobacter pylori, P. aeruginosa – Pseudomonas aeruginosa, S. aureus – Staphylococcus aureus

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Table 3. Estimated free energy of binding (kcal·mol^{-1}) between target proteins and some propolis components

Propolis components/proteins	5LMM	4NX9	5YHG	5FXT
3'4 dihydroxybenzoic acid	–3.76	–5.28	–5.36	–4.16
Chrysin	–5.12	–4.33	–7.19	–5.99
Naringenin	–3.47	–3.63	–6.63	–4.76
Protocatechuic acid	–4.44	–5.23	–4.49	–6.61
Pyrocatechol	–3.08	–5.04	–4.82	–4.06
Rutin	–6.89	–4.13	–4.06	–6.59
Vitexin	–5.22	–5.17	–7.84	–6.84
Vanillic acid	–3.40	–3.17	–4.62	–4.18

5LMM – *E. coli* nife-hydrogenase hydrogen binding, oxidoreductase; 4NX9 – *P. aeruginosa* flagellin protein; 5YHG – *S. aureus* membrane protein; 5FXT – crystal structure of *H. pylori* beta clamp in complex with carprofen

proteins. When the tables are examined, it is noteworthy that the components with high estimated free binding energy form hydrogen (H)-bonds with amino acid residues. In addition, the components generally exhibit polar and hydrophobic interactions. A remarkable situation among the propolis components is that the components containing electronegative atoms contribute to forming H-bonds. Carbon atoms in the components play an active role in polar and hydrophobic interactions. In addition, the aromatic carbons of the compounds exhibit pi-pi (π-π) interaction with amino acid residues.

When the interaction types table was examined, Rutin, the component with the highest antibacterial activity, and the 5LMM target protein were in H-bond, polar, and hydrophobic interaction. This component formed H-bond with amino acid residue CYS17 of the 5LMM target protein. In addition, it interacts polar with GLU22 amino acid residue and hydrophobic with CYS17 amino acid residue. Vitexin also exhibits

Table 4. Interaction types between 5LMM and 4NX9 target proteins and some propolis components

5LMM	H-bonds	Polar	Hydrophobic	π-π
3'4 dihydroxybenzoic acid	CYS17	ARG26	CYS17, CYS19	–
Chrysin	CYS17	THR18	CYS17, CYS19, PRO150	TRP235
Naringenin	–	ARG26	CYS19, PRO150, TRP235	–
Protocatechuic acid	–	ARG26	CYS17, CYS19, CYS149, PRO150, TRP235	
Pyrocatechol	–	ARG26	PRO150, PRO153	TRP235
Rutin	CYS17	GLU22	CYS17	–
Vitexin	CYS17	GLU22	–	–
Vanillic acid	–	SER23, ARG26, ASN236	CYS19, PRO150	–
4NX9	**H-bonds**	**polar**	**hydrophobic**	**π-π**
3'4 dihydroxybenzoic acid	SER95	SER95	VAL115, LEU118, LEU122, ILE303	–
Chrysin	ARG90	ARG90, ASP93	ILE87	–
Naringenin		ARG90, GLU114, GLN117	ILE87, LEU118	–
Protocatechuic acid	–	ARG90	ILE87, LEU118	–
Pyrocatechol	–	–	VAL115, VAL285, ILE288, ILE303	–
Rutin	–	ARG90, ASP93, GLU114, GLN117	ILE87	–
Vitexin	–	ARG90, GLU114, GLN117	ILE87, LEU118	–
Vanillic acid	–	ARG90, GLN117	LEU118	–

5LMM – *E. coli* nife-hydrogenase hydrogen binding, oxidoreductase; 4NX9 – *P. aeruginosa* flagellin protein; H-bonds – hydrogen bonds

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Table 5. Interaction types between 5YHG and 5FXT target proteins and some propolis components

5YHG	H-bonds	Polar	Hydrophobic	π–π
3'4 dihydroxybenzoic acid	TYR129, SER414	SER414	PHE446	PHE446
Chrysin	TYR129	GLN449, ARG454	PRO131, ALA417	TYR129, PHE446
Naringenin	GLN449	TYR129, GLU135, GLN449, TYR458	PRO131	TYR129, PHE446
Protocatechuic acid	–	GLU135	ILE49, PRO131, ALA134	–
Pyrocatechol	SER414	SER414	–	PHE446
Rutin	ASN44, GLN130	ASN44, TYR129, GLN130, GLU135, THR307, GLN413, ARG454	PRO131, ALA134	PHE446
Vitexin	–	ASN44, TYR129, GLN130, GLU135, ASP308, GLN413, ARG454	PRO131, ALA134, MET138	PHE446
Vanillic acid	TYR129, SER414	GLU135, SER414	PHE446	PHE446
5FXT	H-bonds	polar	hydrophobic	π-π
3'4 dihydroxybenzoic acid	THR174	–	LEU179, MET371	–
Chrysin	–	THR174	LEU155, PRO244, ILE249, LEU369	
Naringenin	THR174, LEU179, ILE249	THR174	ILE249, PRO348, LEU369, MET371	–
Protocatechuic acid	–	–	LEU155, LEU179, PRO244, ILE249, LEU369, MET371	–
Pyrocatechol	–	THR174	LEU179, PRO348, LEU369, MET371	–
Rutin	–	THR174	ILE249, PRO348, MET371	–
Vitexin	–	LYS152, THR174	LEU155, PRO244, ILE249, PRO348, LEU369, MET371	–
Vanillic acid	THR174	–	LEU179, PRO348, LEU369	–

5YHG – *S. aureus* membrane protein; 5FXT – crystal structure of *H. pylori* beta clamp in complex with carprofen; H-bonds – hydrogen bonds

similar interactions to the Rutin component. Chrysin component interacts H-bond with amino acid residue CYS17, polar with THR18, and hydrophobically with CYS17, CYS19, and PRO150. This evaluation shows that the atoms in the molecules can be thought to reduce the antibacterial activity depending on the number of hydrophobic interactions with amino acid residues. Interactions with different amino acid residues can alter the inhibition efficiency between the ligand under study and the target biological species. These results can also be seen in Figures 1 and 2, which give the binding modes between the studied components and target proteins.

There are different analytical techniques used for phenolic profiling of propolis worldwide (Bankova et al. 2002; Popova et al. 2004; Watson et al. 2006), but LC-MS/MS recently become a very high sensitivity and the most commonly applied technique alternative

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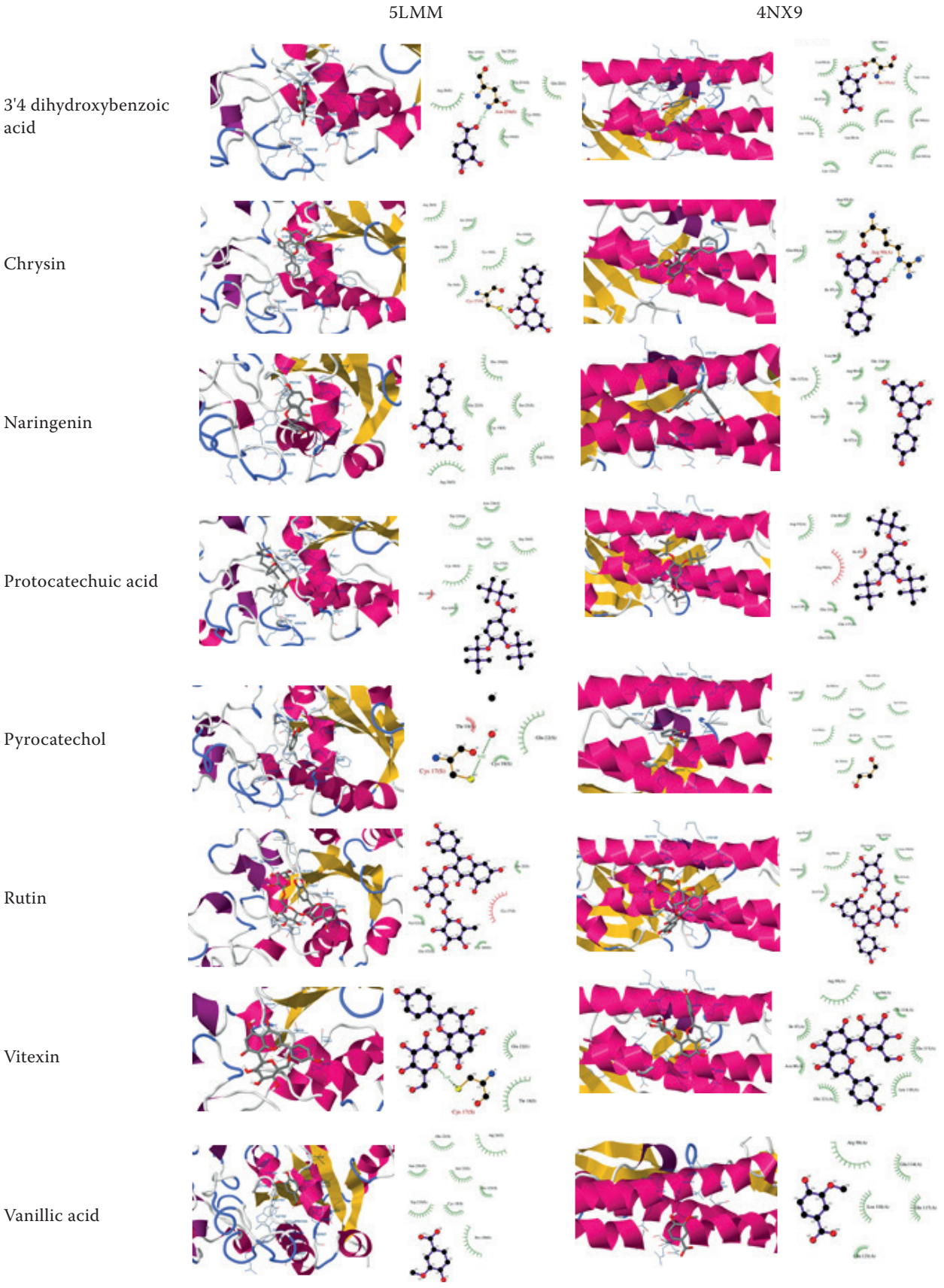

Figure 1. Binding modes between 5LMM and 4NX9 target proteins and investigated components

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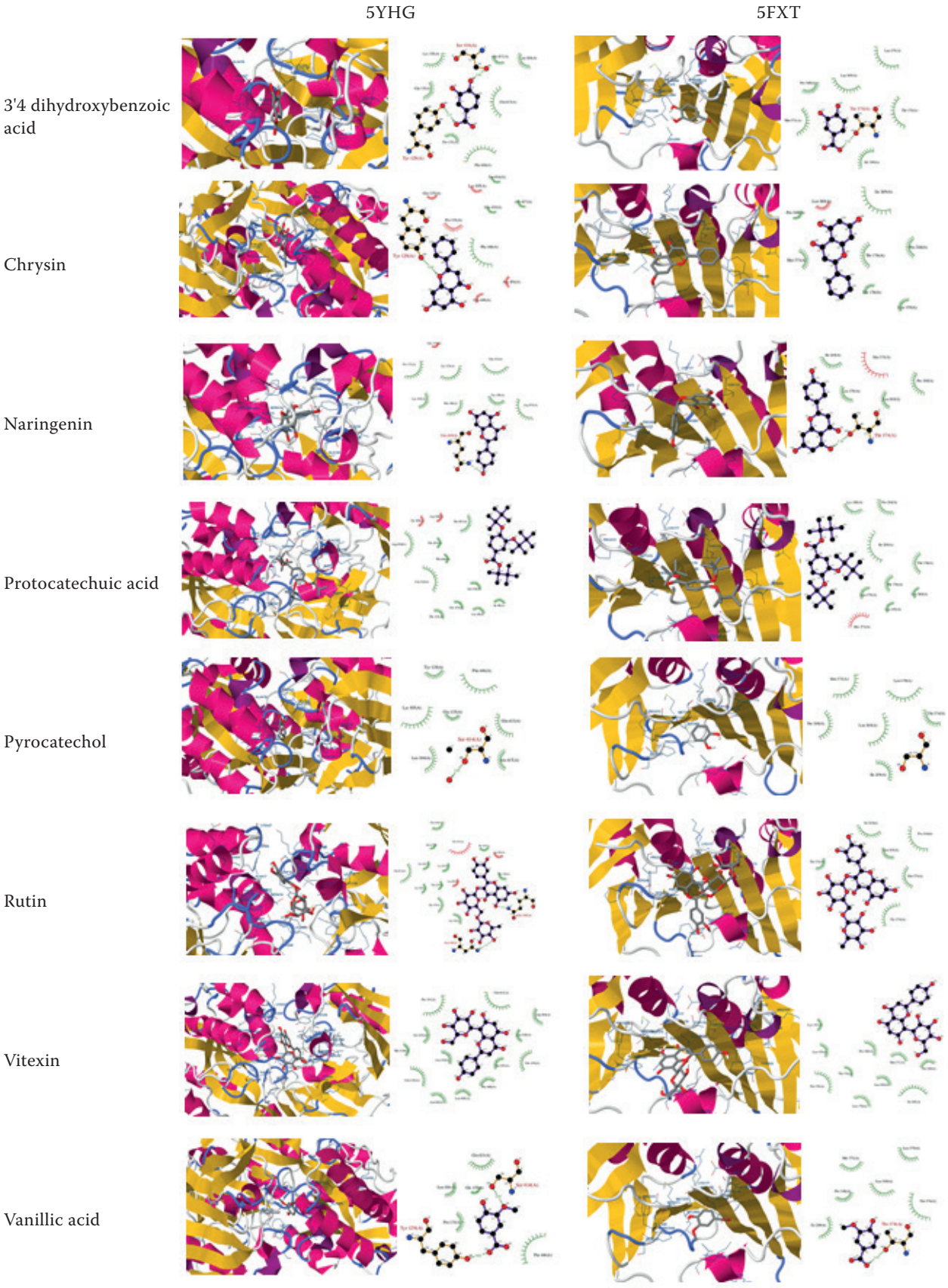

Figure 2. Binding modes between 5YHG and 5FXT target proteins and investigated components

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to traditional methods (Volpi and Bergonzini 2006; Gardana et al. 2007; Pellati et al. 2011). LC-MS has the potential to discover new minor components and gives more structural information that is difficult to determine by traditional methods (Ozdal et al. 2019).

Falcão et al. (2013) demonstrated 62 compounds in Portuguese propolis samples, while Ozdal et al. (2019) reported 32 phenolic compounds in propolis samples obtained from different regions of Türkiye by LC-MS/MS method.

Caffeic acid phenethyl ester (CAPE) is one of the most important compositions related to propolis's high antioxidant activity (Garrido et al. 2012; Ozdal et al. 2019). Contrary to our findings in a study (Ozdal et al. 2019) performed in Türkiye, the highest CAPE value was detected in the propolis samples from the Marmara region. In addition, analysis using LC-MS/MS method indicated that propolis from the Tarsus district of Mersin province included the highest amount of phenolic compounds: phenolic acids [3-4 dimethoxycinnamic acid (620 ppm propolis) and caffeic acid (396 ppm propolis)] and isoflavone [genistein (2 430 ppm propolis)].

In a study performed in Türkiye to search the antimicrobial activity of propolis samples obtained by three various races of bees against some bacteria, researchers exhibited that their propolis samples have higher action against Gram-positive cocci such as *S. aureus*; however had a weak effect against Gram-negative bacteria such as *E. coli* and *P. aeruginosa*, and yeast such as *C. albicans* (Silici and Kutluca 2005). The present study's findings are in agreement with earlier studies (Silici and Kutluca 2005; Przybyłek and Karpiński 2019; Babiker et al. 2020; Sorucu and Ceylan 2021) that found that propolis has a stronger antibacterial activity on gram-positive bacteria.

Strangely, despite a significant number of studies on Brazilian propolis, it was in the middle for both Gram-positive and Gram-negative bacteria (Kim et al. 2011; Dantas et al. 2017). The antibacterial activity of propolis depends on its chemical combination, which differs in countries. In a study conducted by Suleman et al. (2015), most of the ethanolic extracts noticed higher anti-staphylococcal activity from examined 39 propolis samples obtained from South Africa, compared with three propolis samples from Brazil as control, and the MIC value was 6 $\mu g \cdot mL^{-1}$ (Martins et al. 2018). They also confirmed much weaker susceptibility of Gram-negative bacteria such as *E. coli* (MIC value varied from 391 to 1 563 $\mu g \cdot mL^{-1}$) and yeast such as *C. albicans* (MIC value ranged from 98 to 3 125 $\mu g \cdot mL^{-1}$), however very encouraging activity in another pathogenic yeast such as *Cryptococcus neoformans* MIC value between 49 and 391 $\mu g \cdot mL^{-1}$ (Martins et al. 2018).

Our findings concord with a previous report (Babiker et al. 2020) that found that the ethanol extract of propolis has a stronger antibacterial activity on *S. aureus* isolates than that of *P. aeruginosa*, and this may be due to the variations in the genetic compound of isolates.

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CONCLUSION

In conclusion, the biological activities of 3'4 dihydroxybenzoic acid, chrysin, naringenin, protocatechuic acid, pyrocatechol, rutin, vitexin, and vanillic acid ligands at the molecular level, which are propolis components that exhibit antibacterial properties experimentally, investigated by docking studies. Although each component contributed to the antibacterial activity, the contribution of the vitexin component to the antibacterial activity was found to be quite significant.

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