Research in Molecular Medicine

Investigating in Silico and in Vitro Anti-bacterial Activity of Eight Monofloral Iranian Honey Types

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Citation Davazdahemami A, Behbahani M. Investigating in Silico and in Vitro Anti-bacterial Activity of Eight Monofloral Iranian Honey Types. Research in Molecular Medicine. 2022; 10(2):133-142. https://doi.org/10.32598/rmm.10.2.1195

https://doi.org/10.32598/rmm.10.2.1195

Article Type:
Research Paper

Article info:
Received: 7 Mar 2022
Revised: 2 Apr 2022
Accepted: 28 Apr 2022

Keywords:
Antibacterial activity, Iranian honey, Molecular docking, Total flavonoid

ABSTRACT

Background: We have previously reported that monoclonal Iranian honey from different floral sources exhibits a large range of anti-HIV activity owing to the methylglyoxal isolated from honey. This study aims to investigate the antibacterial properties of eight mono-floral Iranian honey samples. Additionally, a significant association between the floral sources and the anti-HIV effects of Iranian honey has been formerly reported.

Materials and Methods: The antibacterial activities of Iranian honey samples were measured using disc diffusion and microbroth dilution methods. The total flavonoid content of each sample was spectrophotometrically evaluated. The best results for the in silico antibacterial activity of the Iranian honeys with the most effective activity in vitro were obtained using the PyRx software, version 0.8. Molecular docking between flavonoids and 6 target proteins (topoisomerase ATPase inhibitor, penicillin-binding protein, D-alanine D-alanine synthase, dihydrofolate reductase, dihydropteroate synthetase, and isoleucyl-tRNA synthetase) was investigated.

Results: The results showed that mono-floral honey from Zataria multiflora and Chamaemelum nobile showed the highest antibacterial effects against *Bacillus subtilis, Streptococcus pyogenes*, *Proteus vulgaris, Bacillus licheniformis*, *Proteus mirabilis,* and *Staphylococcus saprophyticus.* Mono-floral Iranian honey from *Astragalus gummifer*, *Petroselinum* sativum, *Ziziphus mauritiana, Citrus sinensis*, *Nigella sativa*, and *Citrus aurantium* flowers exhibited weak antibacterial activities. However, none of these samples had any effect on *Escherichia coli, Serratia marcescens, Salmonella enterica,* or *Staphylococcus aureus.* Flavonoid contents of *Z. multiflora* and *C. nobile* honey were significantly different from the other mono-floral honey types. The results of the docking study showed that each compound had an appropriate interaction with the targets. The analysis of the docking results showed that flavonoids had the greatest effect on dihydrofolate reductase (3SRW) and D-alanineD-alanine ligase (2ZDQ).

Conclusion: The antibacterial properties of mono-floral honey types are linked to the levels of total flavonoids present, particularly apigenin, quercetin, and kaempferol, which are abundant in certain Iranian honey types. These honey types may be promising candidates for preclinical testing of antibacterial therapies.

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Introduction

Over the past few decades, the efficacy of antibiotics in combating pathogenic bacteria has declined due to the widespread emergence of bacterial resistance; however, there is an increasing interest in screening natural product constituents that have antimicrobial activities. Natural products are potential antibacterial agents [1, 2]. Some important constituents obtained from natural products are alkaloids, flavonoids, coumarins, and triterpenes [3, 4]. Honey is a natural substance that is consumed for its nutritional benefits and positive impact on human health. Its chemical composition and physical properties play an important role in determining its value [5]. Honey contains a variety of secondary metabolites, minerals, proteins, free amino acids, enzymes, and vitamins. Numerous studies have explored the biological activities of honey, such as its antioxidant and antibacterial effects [6]. However, no reports exist on the antibacterial activity of Iranian honey species. Furthermore, computational methods have been employed to identify potential drug targets, with molecular docking being one of the most powerful bioinformatics tools for drug development. This technique has been widely utilized by researchers to investigate the binding affinity of a ligand to a target protein. Molecular docking has proven to be highly effective in the discovery of plant phytochemicals that exhibit antimicrobial properties [7-9]. Previously, we reported that methylglyoxal isolated from honey exhibited potent anti-HIV activity. In addition, a striking correlation is detected between the anti-HIV activity of Iranian honey and floral sources [9]. In the present study, the flavonoid content of Iranian honey types and their antibacterial activities were investigated in vitro and silico.

Materials and Methods

Honey samples

In a prior study [9], we collected mono-floral honey samples from 8 distinct floral sources, namely *Petroselinum* sativum*, Nigella sativa, Citrus sinensis, Zataria multiflora, Citrus aurantium, Ziziphus mauritiana, Astragalus gummifer,* and *Chamaemelum nobile*. The fresh honey samples, each weighing 50 g, were carefully packed and sealed in amber glass bottles and then stored in the dark at 4˚C.

Extraction of total flavonoid compounds

A modified method [10] was employed to extract the total flavonoid compounds from honey samples. Each 50 g honey sample was dissolved in 300 mL acidified distilled water (pH 2.0) at room temperature, and the resulting solution was centrifuged at 3000 rpm for 5 min to eliminate solid particles. The supernatant was mixed with 150 g of Amberlite XAD 2 (pore size 9 nm, particle size 0.3–1.2 mm) for 10 min. The mixture was then transferred to a glass column (25×2.4 cm) and eluted with 300 mL of acidified distilled water (pH 2.0), followed by 300 mL of distilled water to remove all saccharides. The total flavonoids absorbed in the solid phase were eluted with 400 mL methanol and the resulting extract was evaporated to dryness using a rotary evaporator at 40˚C before being stored in a refrigerator [10]. The extract was dissolved in distilled water by agitation and the total flavonoid content was measured using a spectrophotometer at λ=765 nm against the blank.

Bacterial strains

The bacterial strains were purchased from the Iranian Biological Resources Center. The strains utilized in this study were *Bacillus subtilis* (ATCC 6633), *Streptococcus pyogenes* (ATCC 1447), *Proteus vulgaris* (PTCC 1079), *Bacillus licheniformis* (PTCC 1721), *Proteus mirabilis* (PTCC 1076), *Staphylococcus saprophyticus* (PTCC 1440), *Escherichia coli* (ATCC 25922), S*erratia marcescens* (ATCC 1111), *Salmonella enterica* (ATCC 14028), and *Staphylococcus aureus* (ATCC 25923). The bacterial strains were incubated in a nutrient broth medium at the temperature of 37˚C for 12 h.

In vitro antibacterial activity

The antibacterial activity of honey was evaluated using a disk diffusion assay [11]. Bacterial growth was inhibited by honey. The bacterial strains were grown in Nutrient Agar at 37˚C and incubated for 24 h at 37˚C. The zone of inhibition around the disc was measured. To determine the MIC and MBC values, serial dilutions of the honey extract were made and added to a 96-well plate. A nutrient broth was used as the appropriate medium and the inoculum contained approximately 6×10^4 cfu/mL. The plates were incubated for 24 h at 30˚C and the absorbance was read at 620 nm. The MIC was defined as the lowest concentration at which no growth was detected. Also, the MBC was defined as the lowest concentration of the extracts to kill the microorganisms. To determine the MBC value, 50 µL of the wells in which the bacteria was not grown was transferred to a nutrient agar medium. Plates in which the bacteria did not grow and contained the lowest concentrations of extracts were reported as MBC values.

Table 1. The MIC and MBC values of 8 mono-floral Iranian honeys against 10 pathogen bacteria

Pathogen Bacteria	*P. sativum*		*C. sinensis*		*N. sativa*		*C. aurantium*		*Z. mauritiana*		*C. nobile*		*Z. multiflora*		*A. gummifer*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
P. vulgaris	500	1000	0	0	0	0	0	0	0	0	0	0	500	1000	250	0
P. mirabilis	0	0	0	0	0	0	0	0	0	0	500	1000	500	1000	0	0
S.aureus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S.enterica	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. coli	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S. pyogenes	1000	1000	1000	1000	100	250	500	500	500	500	100	100	75	100	500	500
B. licheniformis	1000	1000	250	500	500	1000	1000	1000	1000	1000	50	100	50	100	1000	1000
B. subtilis	500	1000	500	1000	500	100	500	1000	500	1000	75	76	75	100	500	1000
S. marcescens	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S. saprophyticus	0	0	0	0	0	0	0	0	0	0	100	250	100	250	0	0

Table 2. Total flavonoid contents of 8 mono-floral honey types

	P. sativum	*C. sinensis*	*N. sativa*	*C. aurantium*	*Z. mauritiana*	*C. nobile*	*Z. multiflora*	*A. gummifer*
Total Flavonoid (mg/100 g)	0.27	0.29	0.42	0.35	0.37	0.65	0.74	0.32

Docking study

A total of 5 active flavonoids in *Z. multiflora* and *C. nobile* were selected from previous reports, including apigenin (CID: 5280443), luteolin (CID: 5280445), 6-hydroxy luteolin (CID: 5281642), quercetin (CID: 5280343), and kaempferol (CID: 5280863) [12-22]. All compounds were evaluated through molecular docking techniques to determine their ability to inhibit target proteins. Meanwhile, 6 target proteins, including topoisomerase ATPase inhibitor (PDB entry 3TTZ), penicillin-binding protein (PDB entry 3UDI), D-alanineD-alanine ligase (PDB entry 2ZDQ), dihydrofolate reductase (PDB entry 3SRW), dihydropteroate synthetase (PDB entry 2VEG), and isoleucyl-tRNA synthetase (PDB entry 1JZQ) were selected for the docking study in the present research. We obtained the 3D structures of the compounds in the SDF format from the PubChem database [23]. The PDB files containing the 3D structures of the target proteins were obtained from the protein data bank. The PyRx software, version 0.8. was used to perform molecular docking [24]. PyRX includes AutoDock with a Lamarckian genetic algorithm as the scoring algorithm. The docking parameters utilized for the PyRx runs were as follows: 100 docking runs, population size of 150, random starting position and conformation, mutation rate of 0.02, crossover rate of 0.8, and local search rate of 0.06. The docked conformations were clustered by tolerance of 0 Å root mean square deviations.

Statistical analysis

The data were presented as Mean±SD from 3 independent experiments. The analysis of variance was performed to assess the significance between the tested samples and the solvent control. A $P<0.05$ was used as the measure of statistical significance between the samples.

Results

In vitro anti-bacterial activity

The results showed that 2 forms of honey originated from *Z. multiflora* and *C. nobile* showed the best anti-bacterial effect against *B. subtilis, S. pyogenes, P. vulgaris, B. licheniformis, P. mirabilis*, and *S. saprophyticus*. Mono-floral Iranian honey from *A. gummifer, P. sativum, Z. mauritiana, C. sinensis, N. sativa*, and *C. aurantium*

Table 3. The results of the docking study of 6 target proteins with the studied flavonoids

Ligand	Binding Affinity (kcal/mol)	Root Mean Square Deviation/Upper Bound	Root Mean Square Deviation/ Lower Bound
3SRW-apigenin	-9	0	0
3SRW-luteoline	-8.9	0	0
3SRW-6-hydroxy luteolin	-8.8	0	0
3SRW-quercetin	-9	0	0
3SRW-kaempferol	-9	0	0

Ligand	Binding Affinity (kcal/mol)	Root Mean Square Deviation/Upper Bound	Root Mean Square Deviation/ Lower Bound
2ZDQ-apigenin	-7.1	0	0
2ZDQ-luteoline	-9.1	0	0
2ZDQ-6-hydroxy luteolin	-9.1	0	0
2ZDQ-quercetin	-8.8	0	0
2ZDQ-kaempferol	-8.7	0	0

Ligand	Binding Affinity (kcal/mol)	Root Mean Square Deviation/Upper Bound	Root Mean Square Deviation/ Lower Bound
3TTZ-apigenin	-8.8	0	0
3TTZ-luteoline	-8.2	0	0
3TTZ-6-hydroxy luteolin	-8	0	0
3TTZ-quercetin	-8.2	0	0
3TTZ-kaempferol	-7.7	0	0

Ligand	Binding Affinity (kcal/mol)	Root Mean Square Deviation / Upper Bound	Root Mean Square Deviation / Lower Bound
2VEG-apigenin	-6.7	0	0
2VEG-luteoline	-6.7	0	0
2VEG-6-hydroxy luteolin	-6.9	0	0
2VEG-quercetin	-7.2	0	0
2VEG-kaempferol	-6.8	0	0

Ligand	Binding Affinity (kcal/mol)	Root Mean Square Deviation/Upper Bound	Root Mean Square Deviation/ Lower Bound
3UDI-apigenin	-7.9	0	0
3UDI-luteoline	-8	0	0
3UDI-6-hydroxy luteolin	-8	0	0
3UDI-quercetin	-8.4	0	0
3UDI-kaempferol	-8.4	0	0

Ligand	Binding Affinity (kcal/mol)	Root Mean Square Deviation/Upper Bound	Root Mean Square Deviation/ Lower Bound
1JZQ-apigenin	-7.8	0	0
1JZQ-luteoline	-8.2	0	0
1JZQ-6-hydroxy luteolin	-8.5	0	0
1JZQ-quercetin	-8.3	0	0
1JZQ-kaempferol	-8.3	0	0

flowers showed weak antibacterial activities against the mentioned bacteria. The MIC and MBC values of all extracts were estimated and are listed in Table 1. Among these bacterial strains, *S. pyogenes, S. saprophyticus, B. licheniformis, and B. subtilis* were more prone to 2 varieties of honey originating from *Z. multiflora* and *C. nobile* with MIC value of ≤100 µg/mL (Table 1).

Total flavonoid compounds

Total flavonoid compounds of the honey samples are described in Table 2. The highest flavonoid compounds found in honey originated from *Z. multiflora* and *C. nobile* honey kinds with values of 0.65 and 0.74 mg/100 g, respectively (Tables 2). The whole flavonoid contents in mono-floral honey types from *N. sativa, Z. mauritiana, P. sativum, C. sinensis, C. aurantium,* and *A. gummifer were* 0.42, 0.37, 0.27, 0.29, 0.35, and 0.32 respectively (Table 2).

In-silico antibacterial activity

The results of the docking study of the 6 target proteins with the flavonoids are exhibited in Table 3. The results revealed that all the studied compounds had proper interactions with the targets (Figure1-6).

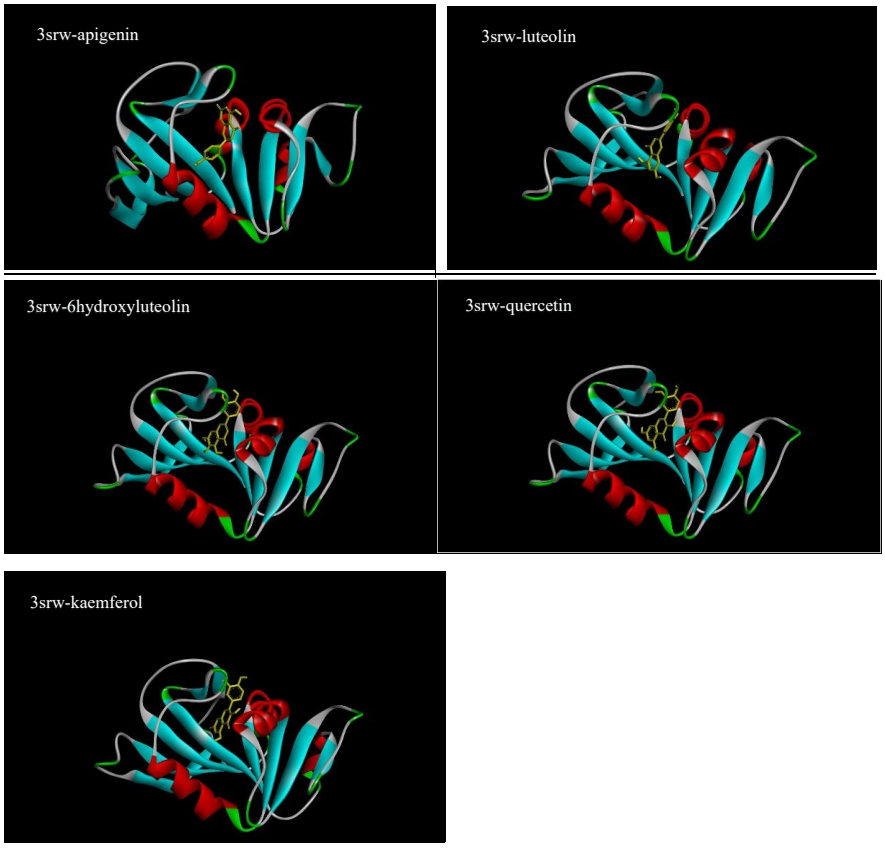

Figure 1. Interaction between 3SRW and ligands

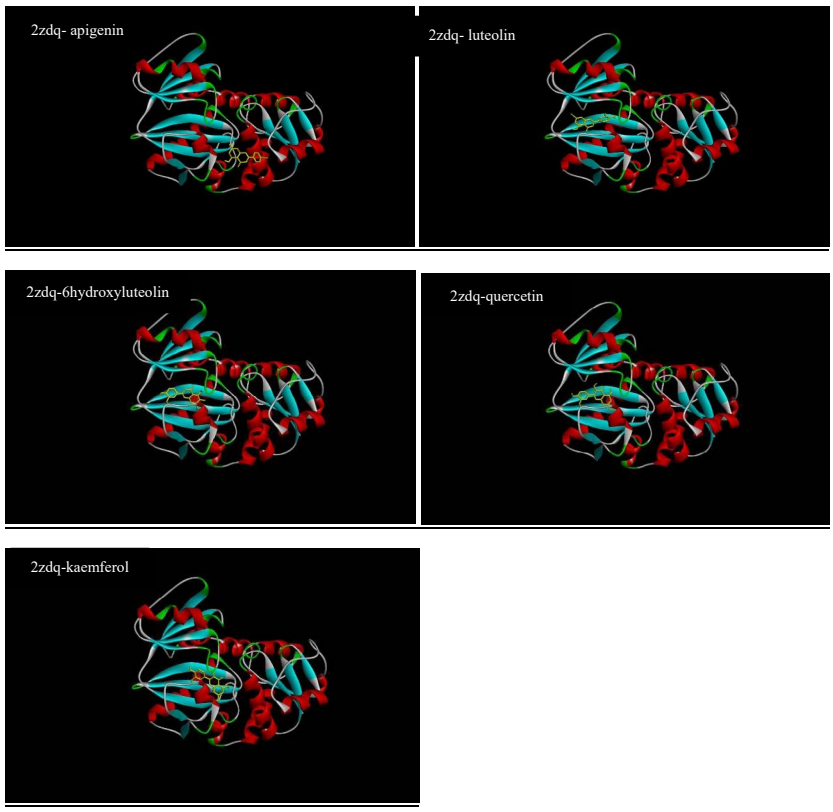

Figure 2. Interaction between 2ZDQ and ligands

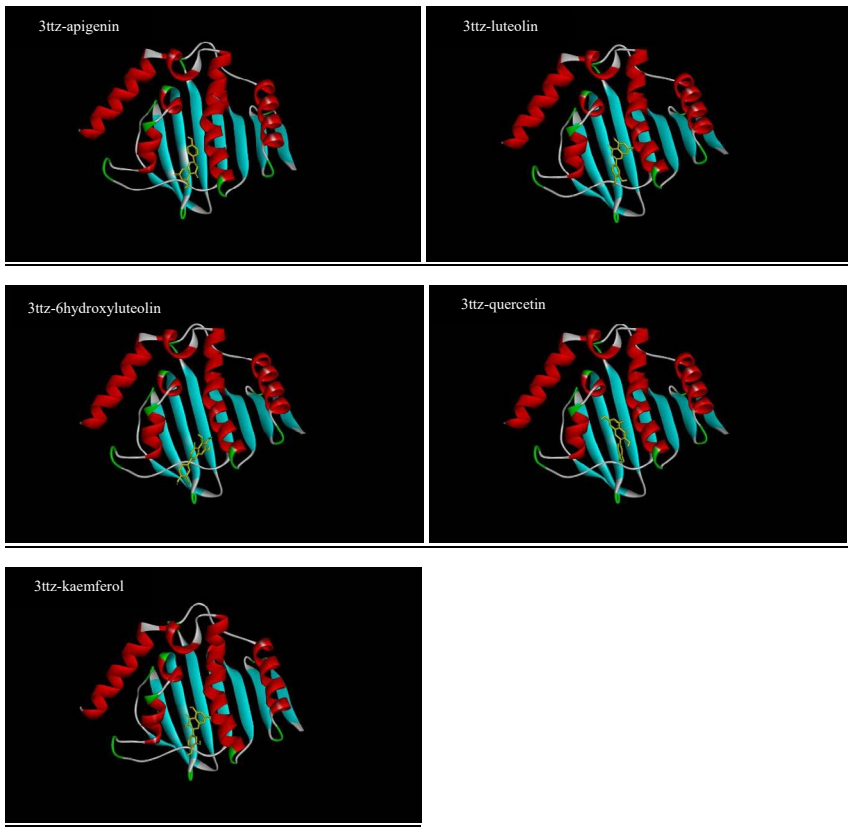

Figure 3. Interaction between 3TTZ and ligands

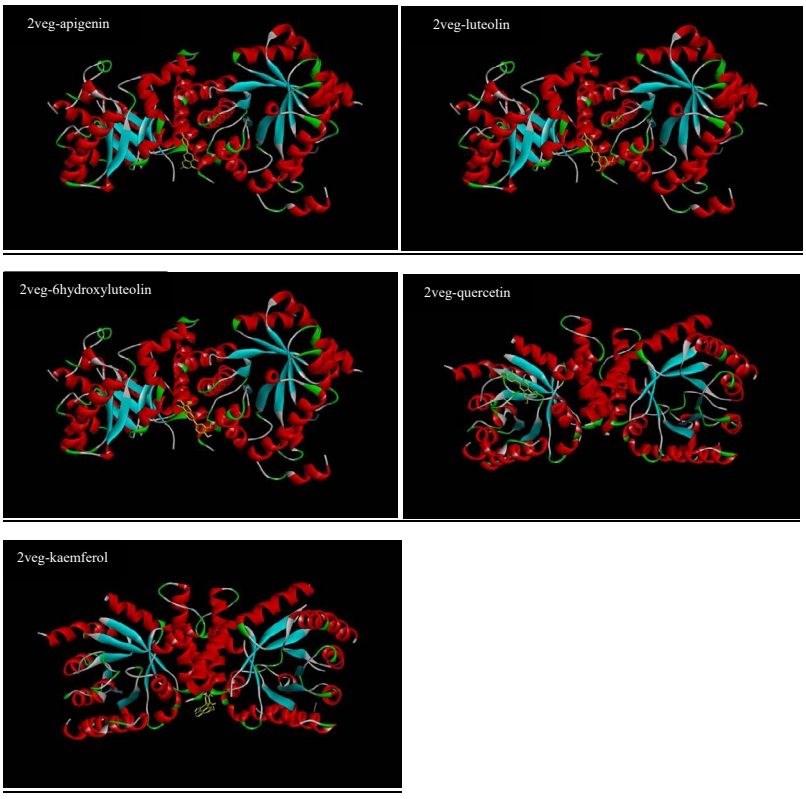

Figure 4. Interaction between 2VEG and ligands

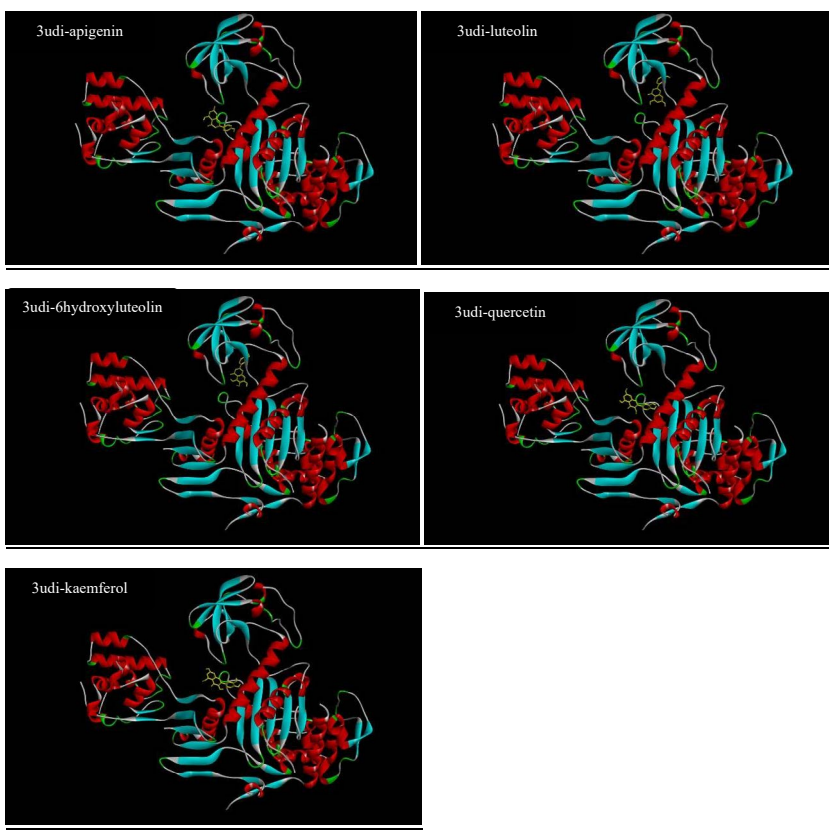

Figure 5. Interaction between 3UDI and ligands

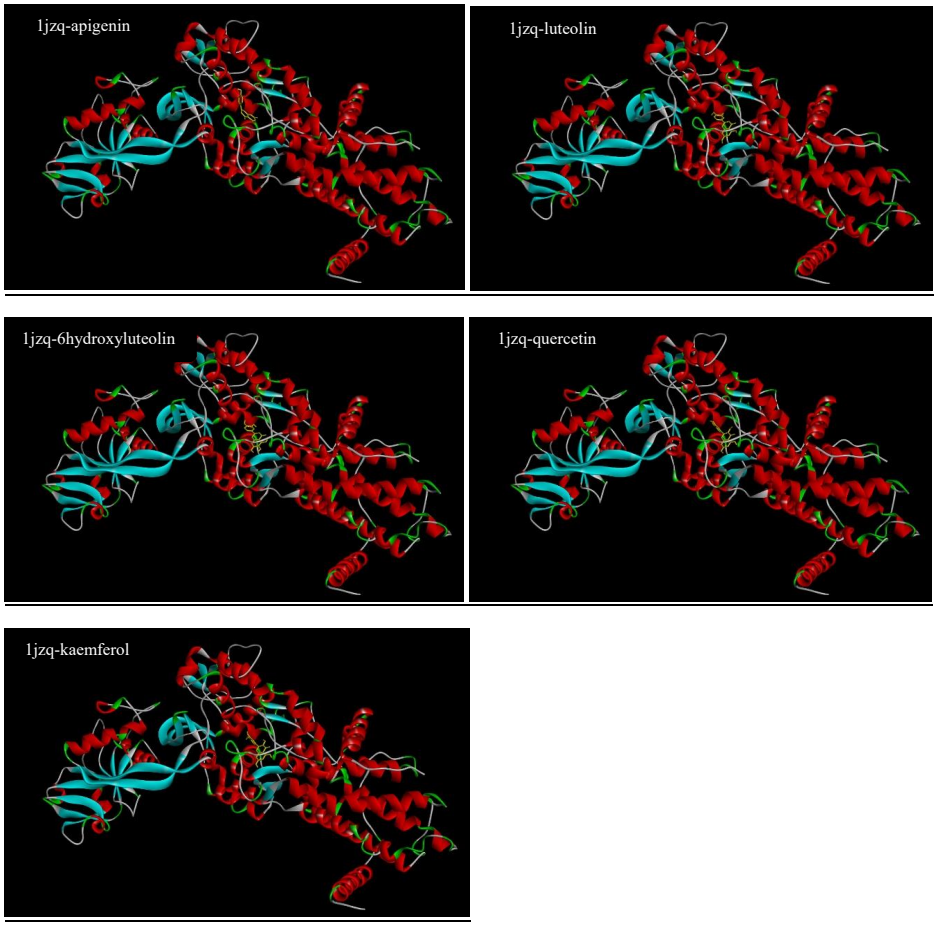

Figure 6. Interaction between 1JZQ and ligands

The results also expressed that each compound had a root mean square deviation less than 2. The analysis of docking outcomes revealed that flavonoids had the best results on 3SRW and 2ZDQ. In supporting these results, apigenin (CID: 5280443), quercetin (CID: 5280343), and kaempferol (CID: 5280863) had the highest efficacy for 3SRW target proteins with a binding affinity in (-9 kcal/mol) for all of them. Also, luteolin and 6-hydroxy luteolin showed the most effective activity against the 2ZDQ target protein.

4. Discussion

According to the given investigations, Iranian honey's antibacterial effectiveness and floral sources are remarkably correlated. In this research, mono-floral honey from *Z. multiflora* and *C. nobile* showed the highest antibacterial effect against *B. subtilis, S. pyogenes*, *P. vulgaris, B. licheniformis, P. mirabilis,* and *S. saprophyticus*; however, mono-floral Iranian honey from *A. gummifer, P. selenum sativum, Z. Mauritiana, C. sinensis, N. sativa,* and *C. aurantium* flowers showed weak antibacterial activity. These findings are often attributed to differences within the secondary compound profile which depends mostly on the floral origin of honey. The docking results also showed that flavonoids had the most effective effect on bacterial proteins, that is 3SRW. In supporting these results, apigenin, quercetin, and kaempferol had the highest efficacy for the 3SRW target proteins. Some information has illustrated that the biological activities of most vital mono-floral honey are associated with their phytochemical composition and distinct earthly sources [25-27]. Our previous study showed a solidarity between the anti-HIV activity of honey and its secondary metabolite content [10]. The current results confirmed the incidence of flavonoid content in mono-floral Iranian honey as a robust antibacterial agent. Some natural flavonoids have been stated to inhibit bacterial activity [28-30]. Several research have proven that different types of flavonoids have antibacterial effects. The apigenin of *P. oleracea* displays antibacterial activity against *Pseudomonas aeruginosa, Klebsiella pneumoniae*, *Salmonella typhimurium*, *P. mirabilis*, and *Enterobacter aerogenes* [31]. Quercetin is a polyphenolic flavonoid with poten-

tial chemoprotective properties that is effective against *S. aureus, E. coli*, *P. vulgaris,* and *P. aeruginosa*. Quercetin has potential as an alternative antibiotic feed additive for animal production [32, 33]. Another study assessed the in vitro antibacterial effects of kaempferol and (−)-epicatechin on *Helicobacter pylori*. The results showed that (−)-epicatechin and kaempferol had antibacterial activities, with (−)-epicatechin being more practical compared to kaempferol [34]. Consistent with these results, our data showed that Iranian honey types with high concentrations of flavonoids, such as apigenin, quercetin, and kaempferol may be promising candidates for the preclinical testing of antibacterial therapies.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles have been considered in this article.

Funding

This study was financially supported by University of Isfahan.

Authors contribution's

Study design, data collection, data interpretation and writing the manuscript: The both authors; Data analysis and final approval: Mandana Behbahani.

Conflict of interest

The authors declared no conflicts of interest.

Acknowledgements

The authors acknowledge the University of Isfahan for providing financial support for this study.

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