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Naringenin, Helichrysin A: Characterization, isolation, molecular docking studies and enzyme inhibitory profiles for carbonic anhydrase, acetylcholinesterase, and *a*-glycosidase

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ABSTRACT: In the current study, Naringenin and Helichrysin A purified and characterized from *Helichrysum plicatum* subsp. *pseudoplicatum*. The inhibitory effects of isolated Naringenin and Helichrysin A were tested against human carbonic anhydrase I (hCA I) and II isoenzymes (hCA II), *a*-glycosidase (α-gly) and acetylcholinesterase (AChE). Naringenin and Helichrysin A's Ki values were found to respectively 51.99±2.78 and 75.75±13.66 nM against hCA I, 36.16±2.02 and 96.81±12.46 nM against hCA II, 0.74±0.04 and 1.27±0.16 nM against AChE, 8.34±1.61 and 9.58±1.90 nM against *a*-gly. As a result, the inhibitory effects of the isolated compounds against each metabolic enzyme examined were demonstrated. Moreover, in the molecular docking study of Helichrysin A, it was observed that the three enzymes had the lowest binding free energy and maximum binding affinity. Helichrysin A and Naringenin show promise as treatments for conditions including epilepsy, leukemia, diabetes mellitus, glaucoma, and Alzheimer's disease.

KEYWORDS: Enzyme inhibition; Helichrysin A; Molecular docking; Naringenin

1. INTRODUCTION

The *Helichrysum* genus belongs to the Asteraceae (Compositae) family and includes 600 species in the world [1]. This genus has 27 taxa in the flora of Turkey, 15 of which are endemic [2]. *Helichrysum* species are generally known as "immortelle, everlasting and golden flower" in the world [3, 4]. In traditional medicine, it is used as a choleretic, hepatoprotective, diuretic, antimicrobial and antispasmodic agent [5]. *Helichrysum* species have phytochemical compounds in the form of flavonoids, chalcones, pyrones, fluoroglucinols, terpenes, essential oils, acetophenone [4, 6]. Many phenolic compounds have been isolated in isolation studies in *H. italicum* [7], *H. graveolens* [8] *H. aureonitens* [8], *H. stoechas* [9], *H. foetidum* [10]. In many pharmacological studies conducted on *Helichrysum* species, anti-diabetic [11], anti-Alzheimer's [12] properties are known. However, for further studies, it is necessary to reveal the pharmacological properties of the isolated compounds that may be responsible for the effect.

Diabetes mellitus (DM) is a chronic metabolic disease characterized by high blood sugar, insulin deficiency, insulin resistance, or both, due to impaired endocrine function [13]. In DM, it is tried to lower blood sugar by various mechanisms. One of them is the inhibition of enzymes such as alpha glycosidase, which converts polysaccharides into monosaccharides [14, 15]. Diabetes causes microvascular complications and causes serious pathological and functional changes in the heart, blood vessels, eyes, kidneys, skin and nerves over time [16]. In order to prevent this, agents that will lower blood sugar are needed. The discovery of the drug metformin with biguanides obtained from *Galega officinalis* has been an incentive for the discovery of new molecules from natural products [17].

Alzheimer's disease (AD) is a neurodegenerative disease that begins with memory loss and can progress to dementia. In the brains of patients with AD, levels of the acetylcholine (ACh) neurotransmitter, which enables nerve impulses to be transmitted from one nerve cell to another, are reduced [15] as well as

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beta-amyloid, which is responsible for nerve cell loss in the hippocampus, accumulates in the brain. The hippocampus is the part associated with memories, learning, emotions. Therefore, these functions cannot be performed over time [18, 19]. Drugs used in neurodegenerative diseases show various side effects such as gastrointestinal problems and liver toxicity [20]. Formation of amyloid protein and the inhibition of the enzyme AChE, which causes the hydrolysis of ACh, show promise in the treatment of Alzheimer's disease [21]. Recently, many plants and the flavonoids isolated from them have come into the limelight for their promising AChE inhibitory effects.

The enzyme carbonic anhydrase (CA) and its isoforms are zinc-containing metalloproteins and catalyze alternating hydration between bicarbonate (HCO^{3-}) and carbon dioxide (CO_2) for pH balance in biological fluids [15, 20]. CAs are involved in various physiological and pathological events such as pH regulation, ion transfer, electrolyte secretion, calcification in bone tissues, biosynthetic reactions such as fat and carbohydrate metabolism, carcinogenesis, secretion of stomach, cerebrospinal fluid and pancreatic fluids. CA inhibitors are widely used as drugs in the treatment of neurological disorders such as epilepsy, glaucoma, cancer, obesity, blood pressure, infection [11, 21-24].

Molecular docking is a technique used to predict how a molecule will bind to a protein receptor, including binding orientation and affinity predictions. This method provides predictive information on how a ligand interacts with the amino acids present at the active region of the enzyme. The primary objective of molecular docking is to determine the optimal binding position of the ligand within the binding site of the protein. It also has a significant impact on the field of drug development, allowing for the screening of extensive chemical libraries for the purpose of identifying potential candidates for drug development [25].

The aim of this study was to evaluate the inhibitory effects of Naringenin and Helichrysin A AChE, *a*-gly, hCA I, and hCA II isolated by chromatographic methods from *H. plicatum* subsp. *pseudoplicatum*. In addition, molecular docking between Naringenin and Helichrysin A isolated with these enzymes determined their binding and affinity. With the findings of this study, the usability of isolated flavonoids in diseases such as glaucoma, AD and DM has been demonstrated.

2. RESULTS and DISCUSSION

2.1. Isolation and structure identification

2 flavonoids HP-1 (Naringenin) and HP-2 (Helichrysin A) were isolated from ethyl acetate extract of *H. plicatum* subsp. *pseudoplicatum* flowers. The structure of the isolated natural compounds (HP-1 and HP-2) was elucidated using 1D- and 2D-NMR.

2.1.1. Compound 1 HP-1 (Naringenin)

^{1}H-NMR (CD_3OD, 400 MHz): δ 7.32 (*dd* J = 1.5 Hz and 8.0 Hz H-2′ and H-6′), 6.95 (*dd*, *J* = 1.5 Hz and *J*= 8.0 Hz, H-3′ and H-5′), 5.97 (*m*, H-6), 5.85 (*m*, H-8), 5.35 (*dd* J= 3.0 Hz, 12.9 Hz, H-2), 3.13 (1H, *dd J* = 17.1 and *J* = 12.9 Hz, H-3_{ax}), 2.70 (*dd J* = 3.0 Hz, *J* = 17. 1 Hz, H-3_{eq}). **^{13}C-NMR,** (CD_3OD, 100 MHz)δ 197.8 (C-4), 164.9 (C-7), 163.4 (C-5), 165.5 (C-9), 159.0 (C-4′), 131.1 (C-1′), 129.1 (C-2′ and C-6′), 116.3 (C-3′ and C-5′), 103.3 (C-10), 97.1 (C-6), 96.2 (C-8), 80.5 (C-2), 44.0 (C-3). When the spectroscopic values of HP-1 compound ^{1}H- and ^{13}C-NMR were compared with the literature, it was confirmed that it is a Naringenin compound [26, 27] (Figure 1).

A B

Figure 1. Structure of HP 1 (Naringenin) (A), HP 2 (Helichrysin A) (B)

2.1.2. Compound 2 HP 2 (Helichrysin A)

^{1}H-NMR (CD_3OD, 400 MHz): *δ* 7.32 (*d*, *J* = 8.48 Hz, H2′ and H6′), 6.83 (*d*, *J* = 8.48 Hz, H3′ and H5′), 6.49 (*d*, *J* = 2.0 Hz, H8), 6.15 (*d*, *J* = 2.0 Hz, H6), 5.34 (*dd*, *J* = 13.02, *J2* = 2.64, *J2* = 2.60 Hz, H2), 4.84 (*d*, *J* = 7.44 Hz, H1″), 3.96 (*d*, *J* = 11.88 Hz, H6″a), 3.80 (*dd*, *J1* = 12.02, *J2* = 5.00, *J2* = 5.00 Hz, H6″b), 3.58–3.44 (*m*, H5″, H3″, H2″), 2.73

(*dd*, *J1* = 17.3 *J2* = 2.84, *J2* = 2.88 Hz, H3). ^{13}C-NMR (CD_3OD, 100 MHz): δ 190.1 (C4), 165.3 (C7), 165.1 (C5), 161.2 (C9), 157.8 (C4′), 129.7 (C1′), 128.0 (C2′ and C6′), 115.2 (C3′ and C5′), 104.3 (C1″), 103.5 (C10), 99.6 (C8), 98.3 (C6), 78.8 (C2), 77.4 (C5″), 77.3 (C2″), 73.5 (C3″), 70.2 (C4″), 61.6 (C6″), 44.9 (C3) (Figure 1). When the ^{1}H- and ^{13}C-NMR spectroscopic values of the HP 2 compound were compared with the literature, it was confirmed that it was Helichrysin A compound [27]. Naringenin and Helicrysin A ((+)-Naringenin-5-*β*-D-glycoside) are flavonoids belonging to the flavanone subclass. Naringenin is abundant in citrus fruits and vegetables such as tomatoes. When the pharmacological activities are examined, many effects such as antioxidant, antidiabetic, anticancer, antimicrobial, anti-inflammatory, immunomodulator, antiadipogenic, hypolipidaemic, antiatherogenic, neuroprotective, memory enhancing are mentioned [28, 29]. In a study on Helichrysin A, it was reported to have an inhibitory effect of advanced glycation endproducts (AGE) formation [30].

2.2. Enzym inhibition assays

The isolated compounds Helichrysin A, Naringenin were evaluated to determine their hCA I, hCAII, AChE and *a*-gly enzyme inhibitory effects. Acetazolamide, tacrine and acarbose were used as the positive controls, respectively [31, 32]. For hCA I, IC_{50} values were determined as 49.51 nM for Helichrysin A, 86.64 nM for Naringenin. For the hCA II, IC_{50} values were determined as 57.76 nM for Helichrysin A, 77.02 nM for Naringenin, The IC_{50} values of acetazolamide were found as 99.0 nM against hCA I iso enzyme and 87.95 nM against hCA II isoenzyme. Concerning the Ki values of the Helichrysin A and Naringenin and acetazolamide as a positive control (Table 1), remarkable effects against hCA I (51.99±2.78; 75.75±13.66; 82.41±10.45) and hCA II (36.16±2,02; 96.81±12.46; 159.60±9.05) respectively, were found. This study is the first to report the inhibitory effect of naringenin against α-glycosidase, AChE, hCA I, and hCA II. Furthermore, it is the first study of *H. plicatum* subsp. *pseudoplicatum* conducted in isolation. Flavanoids are among the most isoform-selective CA inhibitors [33]. In the study conducted by Aydin, Helichrysin A was compared with acetazolamide, and it was reported that it had 2.27 uM for hCA I and 1.27 uM Ki values for hCA II [34] (Table 1 and Figure 2).

The IC_{50} values against AChE were determined as 8.87, 14.15, 5.97 nM, for Helichrysin A, Naringenin, and positive control tacrin, respectively. Helichrysin A and Naringenin effectively with Ki values of 0.74±0.04 and 1.27±0.16 nM, respectively, inhibited the cholinergic enzyme AChE. Also, tacrine as a positive control had a Ki value of 2.43±0.92 nM for AChE (Table 1 and Figure 2). In a study conducted by Liu et al., it was reported that the IC_{50} value of Naringenin against AChE was determined as 3.81 µM [35].

The IC_{50} values against *a*-gly were determined as 16.50, 9.37, 22800 nM, for Helichrysin A, Naringenin, and positive control acarbose, respectively. Helichrysin A and Naringenin had Ki values of 8.34±1.61 and 9.58±1.90 nM towards *a*-gly. The results in the current study show that the isolated Helichrysin A and Naringenin effectively inhibit the *a*-gly enzyme. (IC_{50}: 22800 nM) [32].The results demonstrated that Helichrysin A and Naringenin had a too more efficient Ki than acarbose as a effective starch blocker (Table 1 and Figure 2).

In a study conducted by Acet et al., it was reported that various extracts of *Helichrysum* species were effective against the *a*-gly enzyme at dosage ranges of 3.77-25.42 mmolACAE/g extract and against AChE enzyme at dosage ranges of 0.81-1.48 mgGALE/g extract. In addition, it was evaluated that ethylacetate extracts showed better inhibitory effects against all enzymes. In our study, Naringenin and Helichrysin A were also isolated from the ethyl acetate fraction [36].

Table 1. The inhibition values of Helichrysin A and Naringenin against CA I, CA II AChE and *a*-Gly Enzymes.

Compounds	IC_{50} (nM)								Ki (nM)			
	CA I	r^2	CA II	r^2	AChE	r^2	α-Gly	r^2	CA I	CA II	AChE	*a*-Gly
Helichrysin A	49.51	0.9930	57.76	0.9977	8.87	0.9925	16.50	0.9364	51.99±2.78	36.16±2.02	0.74±0.04	8.34±1.61
Naringenin	86.64	0.9917	77.02	0.9985	14.15	0.9925	9.37	0.9898	75.75±13.66	96.81±12.46	1.27±0.16	9.58±1.90
Acetazolamide[a]	99.00	0.9825	87.95	0.9940	-	-	-	-	82.41±10.45	159.60±9.05	-	-
Tacrine[b]	-	-	-	-	5.97	0.9706	-	-	-	-	2.43±0.92	-
Acarbose[c]	-	-	-	-	-	-	22800	0.9922	-	-	-	-

[a]Positive control for CAI and CA II inhibition [31]
[b]Positive control for AChE inhibition [31]

[c]Positive control for α-gly inhibition [32]

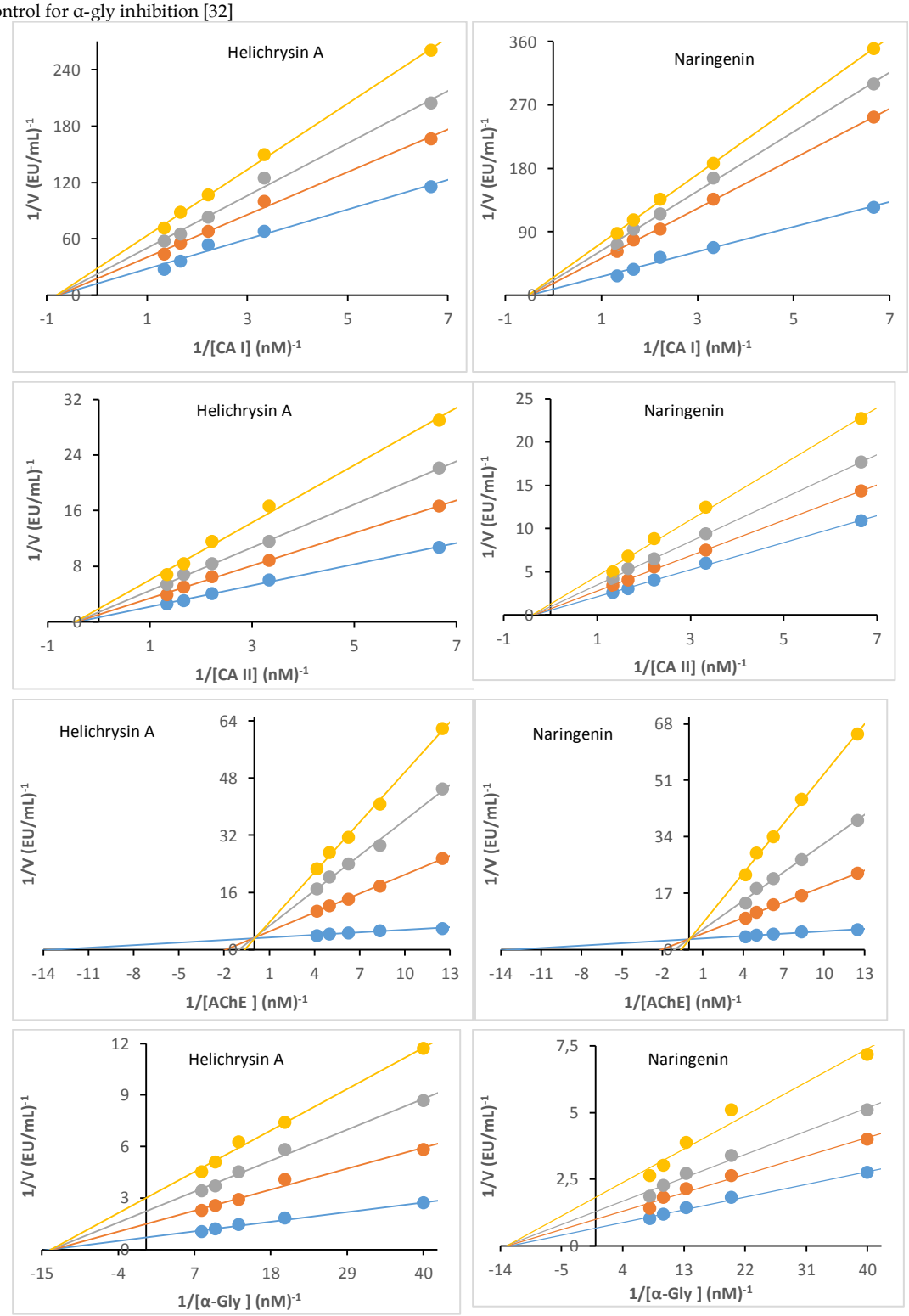

Figure 2. Lineweaver-Burk graphs of Helichrysin A and Naringenin towards CA I, CA II, AChE, and α-Gly enzyms

2.3. Moleculer docking

In the current study, the inhibition effects of isolated Naringenin and Helichrysin A compounds on CA I, CA II, AChE and *a*-gly were evaluated. The mechanisms of binding of Naringenin and Helichrysin A, which have high inhibitory effects against CA, *a*-gly and AChE enzymes, to the active sites of the enzymes were evaluated by Molecular docking, an *in silico* study. In order to forecast ligand binding affinities to proteins and to examine potential interactions between ligands and proteins, molecular docking studies are

essential to the drug development process [37]. The free energy of binding is a commonly used metric to determine ligand affinity for proteins. A medicine is said to have a high binding activity against an enzyme when it binds to it with a low binding free energy [38, 39]. In our study, the relationship between Naringenin, Helichrysin A and related enzymes were evaluated as molecular docking study *in silico*. The *in silico* results in Table 2 and Figure 3 show that, among the isolated compounds, Helichrysin A had the lowest binding free energy and the maximum binding affinity for CA I (G: -7.367 kcal/mol), CA II (G: -7.760 kcal/mol), AChE (G: -13.384 kcal/mol). Among the other isolated compound, Naringenin had the most binding affinity for the α-glycosidase enzyme (G: -9.608 kcal/mol). These findings are also compatible with the inhibiton values in Table 1. The interaction between Helichrysin A and the active region of the hCA I enzyme can be characterized by polar and non-polar interactions and hydrogen (Hyd) bonds between the OH groups of the structure and the amino groups Thr199, Pro201 and Gln92. The interaction between Naringenin and the active region of the hCA I enzyme can be characterized pi-pi stacking interactions between His94 amino acid. The interaction between Helichrysin A and the active region of the hCA II enzyme can be characterized by polar and non-polar interactions and Hyd bonds between the OH groups of the structure and the amino groups Asn67, Asn 62 ve Thr199 and pi-pi stacking interactions between Phe131 amino acid. Naringenin and the active region of the hCA II enzyme can be characterized by polar and non-polar interactions and Hyd bonds between the OH groups of the structure and the amino groups Asn67 and Thr199 the interaction between Naringenin and the active region of the hCA II enzyme can be characterized pi-pi stacking interactions between His94 amino acid. The interaction between Helichrysin A and the active region of the AChE enzyme can be characterized by polar and non-polar interactions and Hyd bonds between the OH groups of the structure and the amino groups Arg289, Gly119, Ser200, Glu199, His440 and pi-pi stacking interactions between Phe330 amino acid. Naringenin and the active region of the AChE enzyme can be characterized by polar and non-polar interactions and Hyd bonds between the OH groups of the structure and the amino groups Phe288, Arg289 the interaction between Naringenin and the active region of the hCA II enzyme can be characterized pi-pi stacking interactions between Phe330 ve Tyr121 amino acid. The interaction between Helichrysin A and the active site of the α-glycosidase enzyme can be characterized by polar and non-polar interactions and Hyd bonds between the OH groups of the structure and the amino groups Thr 205, Gln603, Asp327. Naringenin and the active region of the *a*-gly enzyme can be characterized by polar and non-polar interactions and Hyd bonds between the OH groups of the structure and the amino groups Tyr299, Asp327, Gln603 (Table 2, Figure 3).

Table 2. Binding affinity (kcal/mol) of the isolated compounds (Naringenin, Helichrysin A) to CA I, CA II, AChE and α-Gly enzymes

	Docking Score	XP GScore	Glide GScore	Glide Emodel
	Carbonic Anhydrase I (PDB: 4WR7)			
Naringenin	-4.922	-4.940	-4.940	-46.008
Helichrysin A	-7.360	-7.367	-7.367	-54.546
Acetazolamide	-6.359	-7.281	-7.281	-50.465
	Carbonic Anhydrase II (PDB: 5AML)			
Naringenin	-4.917	-4.935	-4.935	-49.827
Helichrysin A	-7.753	-7.760	-7.760	-59.122
Acetazolamide	-5.927	-6.849	-6.849	-50.340
	Acetylcholinesterase (PDB: 4TVK)			
Naringenin	-9.679	-9.697	-9.697	-50.191
Helichrysin A	-13.376	-13.384	-13.384	-55.690
Tacrine	-12.968	-12.968	-12.968	-59.064
	α-Glycosidase (PDB: 3L4Y)			
Naringenin	-9.600	-9.608	-9.608	-68.098
Helichrysin A	-6.366	-6.366	-6.366	-35.898
Acarbose	-16.526	-16.854	-16.854	-98.119

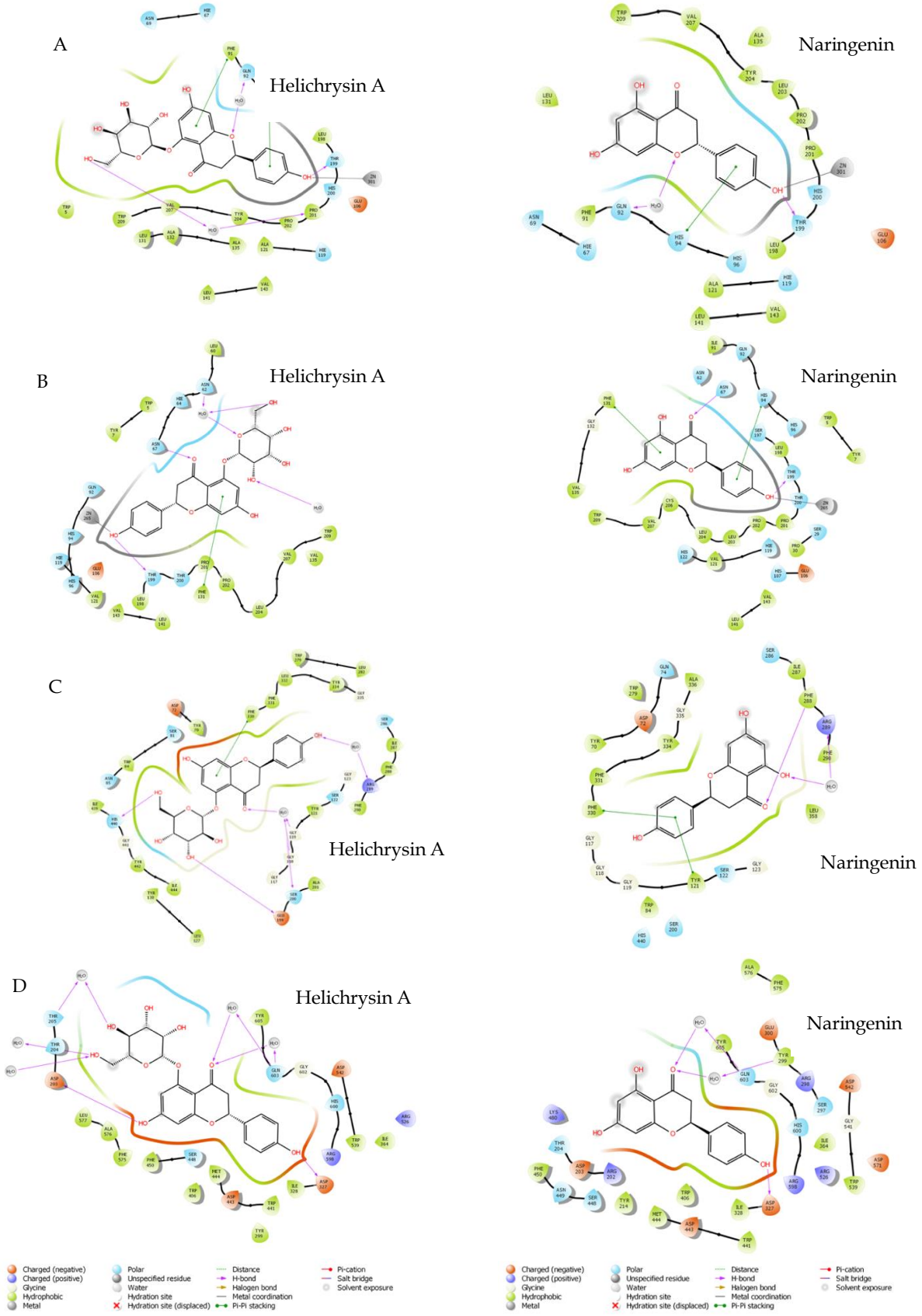

Figure 3. Interaction of the ligands with the key amino acids within the binding site of A: hCA I (PDB id: 4WR7). B: hCA II (PDB id: 5AML). C: AChE (PDB id: 4TVK). D: *α*-Gly (PDB id: 3L4Y)

4. CONCLUSION

Plants produce secondary compounds in nature to protect themselves and continue their generation. These secondary compounds have extremely important biological activities. The activities of Naringenin and Helichrysin A compounds isolated and characterized from the *H. plicatum* subsp. *pseudoplicatum* plant against CA I and II, AChE and glycosidase enzymes were evaluated, and their effects on the enzymes were also evaluated *in silico*. *In vitro* and *in silico* studies are compatible with each other. These natural compounds are promising for use in the pharmaceutical industry. In future studies, its antidiabetic, antiglaucoma and anti-Alzheimer effects should also be investigated *in vivo*.

5. MATERIALS AND METHODS

5.1. Plant Material

H. plicatum DC. subsp. *pseudoplicatum* flowers, was collected by Leyla Güven in Köşk village-Erzurum- Turkey (altitude 1950 m) and was authenticated by Prof. Dr. Yusuf Kaya. A voucher specimen was left at Atatürk University Erzurum Biodiversity Application and Research Center (No. AEF 1374).

5.2. General Experimental Procedure

1D (^{1}H-NMR, ^{13}C-NMR) NMR spectra were derived using Varian Mercury Plus (400 MHz for 1H-NMR and 100 MHz for ^{13}C-NMR) spectrometers. Solvents were obtained from Sigma-Aldrich (USA) and silica gel 60 (0.063–0.2 mm) (Merck, Germany) was used for open column chromatography (CC). Precoated silica gel 60 F254 plates (Merck) were used for TLC. The spots were sprayed with 1% vanillin solution in strong sulfuric acid, then heated to 110 °C to make them visible. For the purpose of conducting bioactivity tests, all commercially available reagents were acquired from Sigma-Aldrich (USA).

5.3. Extraction and Isolation Studies

400 g of powdered capitulum was extracted 3 times for 3 days with a mechanical mixer at 40 ^{o}C with 5 L of methanol. After each extraction process, the extracts were filtered, the filtrates were combined and condensed in rotavapor, at 40 ^{o}C at 120 rpm. The resulting 100 g methanol extract was dissolved with 2 liters of H_2O:MeOH, 9:1 mixture and taken to a 3 t separation funnel. On the dissolved extract, each process was separated into its fractions using 500 mL of n-hexane, dichloromethane and ethyl acetate sequentially, to be repeated 10 times. After the depletions, the extracts were filtered, the filtrates were combined and condensed in rotavapor, at 40 ^{o}C at 120 rpm. After condensation, n-hexane, dichloromethane, ethyl acetate, and water extracts were obtained at yields of 2.1%, 0.35%, 7.1%, 16.72%, respectively. Thin layer chromatography (TLC), open column chromatography (CC), high pressure liquid chromatography (HPLC) were used in isolation studies. The isolation process is shown in detail in Figure 4. In the isolation studies performed on the ethyl acetate extract of *H. plicatum* subsp. *pseudoplicatum* flowers, the compounds we coded as HP 1 (Naringenin) and HP 2 (Helichrysin A) were obtained purely.

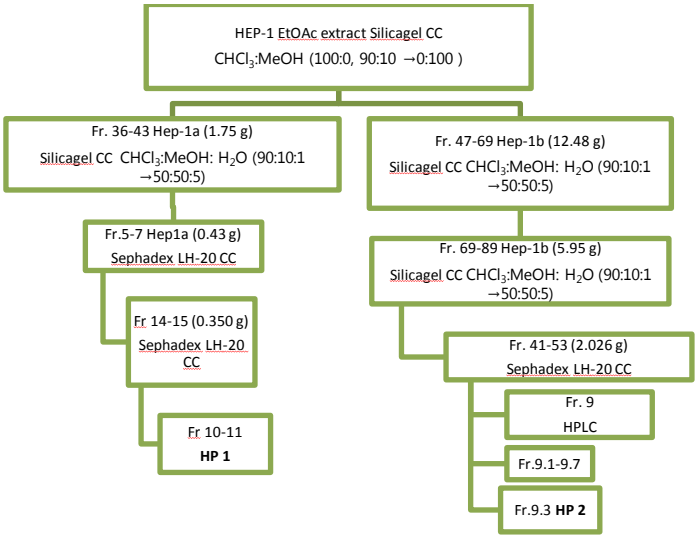

Figure 4. Isolation scheme *of H. plicatum* subsp. *pseudoplicatum* capitulum

5.4. Enzym Inhibition Assays

5.4.1. Determination of AChE Inhibition Effects

The effects of naringenin and Helichrysin A on AChE inhibition were determined spectrophotometrically [40] the Ellman method [41]. Acetylcholine iodate (AChI) and 5,5'-Dithio-bis(2-nitro-benzoic) acid (DTNB) are substrates used in the enzymatic reaction. 100 M, 50 µL of Tris/HCl buffer (pH 8.0), 390 µL of sample, and 10 µL of AChE were mixed and kept in the dark for 10 min at room temperature. Then, 25 µL of each of the solutions containing the substrates was added. In the mixture, DTNB reacts with thiocolin, which is a breakdown product, resulting in 5-thio-2-nitrobenzoic acid, which has a yellow color. The absorbance of the resulting yellow color at a wavelength of 412 nm is recorded.

5.4.2. Determination of a-Gly Inhibition Effects

The enzyme inhibitory effect of naringenin and Helichrysin A *a*-gly was performed using p-nitrophenyl-D-glycopyranoside (p-NPG) substrate, according to the method used by Tao et al. [42]. First, 50 µL of phosphate buffer (pH 7.4), 10 µL of enzyme solution (0.15 U/mL), and 20–100 µL of sample were mixed. Then, substrate was added and incubated at room temperature to start the reaction. Absorbances were measured spectrophotometrically at 405 nm.

5.4.3. Determination of CA I and II Isoenzyme Inhibition Effects

hCA I and II enzyme inhibition effect assay of Naringenin and Helichrysin A was performed according to the method of Şenol et al. [43]. hCA I and hCA II isoenzymes were obtained by centrifugation and purification of the resulting serum of human erythrocytes using Sepharose-4B-L-Tyrosin-sulfanilamide affinity chromatography technique. The obtained elutes were recorded at 280 nm by controlling the spectrophotometer. In the enzyme inhibition effect experiment, p-nitrophenolate was used as a substrate. The absorbance differences of the p-nitrophenolate ion formed as a result of the reaction in 3 minutes were determined by spectrophotometric measurements at 348 nm [44].

5.4.4. Inhibition Kinetics

The isolated compounds and substrate concentrations were used to examine the in vitro inhibition mechanisms of Naringenin and Helichrysin A. IC_{50} and Lineweaver-Burk curves [44] in enzyme inhibition were calculated and graphed taking into account previous studies [45, 46]. IC_{50} and Ki values of Naringenin and Helichrysin A were calculated from the data obtained, and the inhibition types of the enzymes studied were determined as in previous studies [47].

5.4.5. Molecular Docking Studies

The interactions of Naringenin and Helichrysin A with hCA I, hCA II, AChE, *a*-gly enzymes were determined using molecular docking method. For this purpose, in the first stage of molecular docking studies, molecular structures were created with smiles codes from the Drugbank internet database, and utilizing Maestro's LigPrep program, optimization experiments were conducted. (S. Schrödinger Version 2020-3: LigPrep, LLC, New York, NY2020). Enzyme structures were obtained from the RCSB Protein Data Bank website ((PDB IDs; hCA I: 4WR7, hCA II: 5AML, AChE: 4TVK, *a*-Gly: 3L4Y). Possible interactions and binding affinities of the molecules with each enzyme were determined by the molecular docking program Schrödinger Molecular Modeling Suite (MMshare 5.1.139; Maestro 12.5.139 Versions).

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