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Enzyme Inhibition Properties of *Calendula officinalis, Matricaria chamomilla,* and *Anthemis pseudocotula*: Kinetics and Molecular Docking Studies

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Abstract: This study determined the enzyme inhibition potential of three species (*Calendula officinalis, Matricaria chamomill*a, and *Anthemis pseudocotula*) from the Asteraceae family through *in silico*, followed by *in vitro* studies. Quinic acid, fumaric acid, gallic acid, chlorogenic acid, vanillic acid, quercetin, apigenin, and isorhamnetin were determined by LC-MS/MS in all of the species. Metabolic enzymes are essential catalysts regulating biochemical reactions within living organisms, facilitating energy production, detoxification, and biosynthesis. These enzymes play a crucial role in maintaining cellular homeostasis and are tightly regulated to ensure optimal metabolic function. High docking scores were also obtained for butyrylcholinesterase (BChE), α-glycosidase, α-amylase, and human carbonic anhydrase I and II enzymes (hCA I and hCA II). Among the extracts, *Anthemis pseudocotula* was concluded to be the best inhibitor for the enzymes, which was further determined by in vitro enzyme inhibition tests. Besides, it was concluded that all extracts showed anti-cholinergic, anti-diabetic, and anti-glaucoma properties. This is the first study determining the enzyme inhibition property of *Anthemis pseudocotula* and the three species' hCA I and hCA II inhibition activities.

Keywords: Enzyme inhibition; *Matricaria chamomilla; Anthemis pseudocotula; Calendula officinalis*; LC-MS/MS

1. Introduction

The world's modern medicine strategy has started evolving to natural products as they are rich in bioactive compounds with several health benefits, safe with fewer side effects, non-toxic, and easily available at affordable prices [1-3]. According to the WHO, at least 25% of drugs available are derived directly or indirectly from plants. Rather than plants being rich in primary metabolites like carbohydrates, proteins, and lipids, which enrich their nutritional value, secondary metabolites of the plants, such as alkaloids, phenolics, terpenes, and flavonoids, provide ecological privileges to them, thus, medicinal benefits to mankind [4,5]. Among plant families, Asteraceae, Lamiaceae, Apiaceae, Fabaceae, Apiaceae, Rosaceae, and Ranunculaceae are reported to be the most important medicinal plant

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families supported in local studies. It was reported that the ten medically important medicinal plants in Thailand are also listed as important in the world: Asteraceae, Leguminosae, Rutaceae, Lamiaceae, Malvaceae, Solanaceae, Apocynaceae, Euphorbiaceae, Araliaceae, Anacardiaceae. The Asteraceae family is one of the largest families with more than 1,900 genera and 32,000 species, and has been used in traditional medicine and as functional food since ancient times [6]. In 2015, the Nobel Prize in Physiology or Medicine was awarded for the discovery of artemisinin and avermectin, of which the former originates from the genus Artemisia *(*Asteraceae family), fundamentally changing the treatment of parasitic diseases around the globe [7]. Avermectins had inhibitory effects on lactoperoxidase from bovine milk [8], bovine carbonic anhydrase enzyme [9], and goat liver carbonic anhydrase enzyme [10]. *Matricaria chamomilla* from daisies has been used in Traditional Chinese Medicine for years to treat stomach problems, cramps, dermatitis, and minor infections and as a remedy for diarrhea in Nepalese medicine [11]. Similarly, tonics from *Cichorium intybus* have also been used to treat enlarged spleen and fever in Indian Ayurveda medicine, and a decoction from leaves was used as a cure for rheumatism and gout. *Bidens pilosa* is used as a remedy for liver problems and to lower blood pressure and is a major ingredient in herbal infusions in Taiwanese folk medicine [12].

The application of plants in folk medicine does not guarantee their place as a drug candidate. Even though the diverse phytoconstituents of plants provide a pool full of active ingredients, it is common to observe plants' poisonous and toxic properties [13-16]. This may lead to a decline in the preference for plants instead of synthetic analogs with a simple structure and fast effect [17,18]. Therefore, high-throughput studies need to be conducted regarding quality, safety, standardization, efficacy, toxicology, and proper understanding of the evolution of modern medicine to plant-derived drugs once the biological effect is determined to replace synthetic drugs with natural and more effective counterparts [19-21]. In this study, three different genera from the Asteraceae family, namely, *Calendula officinalis*, *Matricaria chamomilla*, and *Anthemis pseudocotula*, were investigated for their potential to be drug candidates through enzyme inhibition studies. Even though these species have been well-known for their health benefits for centuries, limited studies have been conducted to introduce them into modern studies [22-27]. For this purpose, ethanol extracts of the samples were prepared and subjected to LC-MS/MS analysis to determine phytochemical constituents. Then, major compounds detected in the extracts were selected for *in silico* docking studies to foresee the potential of enzyme inhibition mechanisms. *In vitro* enzyme inhibition assays were performed to confirm the docking results, conclude the consistency of *in silico* and *in vitro* studies, and determine IC_{50} values against metabolically important enzyme targets, including AChE, BChE, α-glycosidase, α-amylase, hCA I, and hCA II.

Alzheimer's disease (AD), which is currently seventh on the top ten causes of death list reported by the World Health Organization (WHO), still suffers from a lack of completely safe and effective treatment [28-30]. Currently applied strategies are defined regarding the stage of the disease and target the symptomatic treatment of the patients. Cholinesterase inhibitors, such as Aricept® (Donepezil), Razadyne® (Galantamine), and Exelon® (Rivastigmine), are FDA-approved drugs for AD patients with mild to moderate cognitive deterioration and improve the quality of life for both AD dementia patients, temporarily [31-33]. Gastrointestinal symptoms and sleep disorders are common adverse effects of cholinesterase inhibitors, typically affecting 5–20% of patients [34-36]. This increases the demand for better drugs with fewer side effects. Since plants contain a wide variety of bioactive compounds, they have a good potential to be a source of therapeutics [37,38].

Diabetes is also in the eighth of the top ten causes of death list reported by the WHO, affecting over 400 million people globally. Many strategies have been developed to combat the diseases, including pharmacological and non-pharmacological, but the required success has not been achieved [39-41]. Pharmacological approaches include blood glucose level decreasing agents introduced to the body and improved lifestyle [42]. These decreasing agents are insulin, insulin-like growth factors, insulin analogs, insulin secretagogues, antihyperglycemics, insulin sensitizers, glucose reabsorption inhibitors, and alpha-glucosidase inhibitors [43-45]. α-Glycosidase inhibitors (AGI) are considered a reasonable option as a first-line drug in the treatment of patients with type-2 diabetes mellitus (T2DM) as they specifically target postprandial hyperglycemia, a possible independent risk factor for cardiovascular complications [46,47]. Acarbose, Miglitol, and Voglibose are the clinically used AGIs that act by delaying the digestion of complex carbohydrates. This mechanism of action causes a decrease in BGL as well as osmotic effects. The disaccharides that are not digested may lead to side effects such as diarrhea,

flatulence, and abdominal pain [48,49]. Therefore, research on new AGIs with fewer side effects is in progress [50].

Carbonic anhydrases (CA) have a regulatory role in many physiological processes such as gluconeogenesis, ureagenesis, fluid secretion, acid/base balance, gastric acid production, and transport of CO_2 from tissues to the lungs (in the form of bicarbonate) through the blood [51-53]. The critical importance of CAs in regulating these processes is that they play a major role in the pathophysiology of various diseases like hemolytic anemia, glaucoma, renal tubular acidosis, osteoporosis, neuropathic pain, and colorectal cancer [54]. As a result, carbonic anhydrase inhibitors (CAIs) find therapeutic applications for the treatment of various clinical disorders [55-59]. Scientists have identified sixteen isoforms of CAs, each of which plays a unique role in treating various diseases [60]. For instance, while CA I and CA II are linked to hemolytic anemia, CA II is also associated with glaucoma, epilepsy, edema, and altitude sickness [61]. Many diseases, from cancer to diabetes, have been linked to different CA isoforms. Therefore, it is important to identify and determine novel CAIs.

2. Experimental

2.1. Plant Materials and Chemicals

C. officinalis, *M. chamomilla*, and *A. pseudocotula* used in this study were collected from Kayadibi village, Dalaman, at an altitude of 350–400 meters under the supervision of the Dalaman Agricultural District, Türkiye. Plant Taxonomist Prof. Dr. Mehmet Fidan registered the species to the Siirt University Herbarium with fixture numbers SUFAF5021, SUFAF5022, and SUFAF5023 for *M. chamomilla*, *A. pseudocotula*, and *C. officinalis*, respectively. α-Amylase from *Bacillus sp.* 1,500 units/mg protein, acetylcholinesterase from electric eel (*Electrophorus electricus*) Type VI-S, lyophilized powder, 200-1,000 units/mg protein, acetylcholine iodide, butyrylcholinesterase from equine serum 500 U/mg, butyrylcholine iodide, and 5,5′-dithio-*bis*-(2-nitrobenzoic acid) (Ellman's reagent), Reference phytochemical standards are of analytical grade, and (quinic acid, gallic acid, fumaric acid, epigallocatechin, gentisic acid, catechin, chlorogenic acid, tannic acid, protocatechuic aldehyde, epigallocatechin gallate, epicatechin, 4-OH-benzoic acid, vanillic acid, syringic acid, caffeic acid, vanillin, syringaldehyde, epicatechin gallate, ferulic acid, sinapic acid, p-coumaric acid, coumarin, cynaroside, salicylic acid, rutin, miquelianin, isoquercitrin, o-coumaric acid, hesperidin, genistin, ellagic acid, rosmarinic acid, cosmosiin, astragalin, quercitrin, nicotiflorin, daidzein, fisetin, quercetin, luteolin, naringenin, hesperetin, apigenin, genistein, kaempferol, chrysin, amentoflavone, and acacetin) were purchased from Sigma Aldrich (Steinheim, Germany).

2.2. Preparation of Daisy Samples

Preparation of the extracts was conducted according to previous studies [63,64]. Ethanol extracts of daisies were prepared by weighing 10 g of dried flowers after grinding the pieces up to 0.5-10 mm. Then, 50 mL of ethanol was added to the milled flowers in a beaker, and the samples were mixed for 5 hours. Ethanol was removed from the extracts by rotary evaporator, 100 mg of dry extracts were separated for LC-MS/MS analysis, and 1 mg/mL of DMSO stock solutions of each extract were prepared for enzyme inhibition analysis and then diluted to working standards with distilled water.

2.3 Screening of Secondary Metabolites of Daisies by LC-MS/MS

Screening of secondary/polyphenolic metabolites of daisies by LC-MS/MS had been reported previously [65]. Thirty-five OAs and PCs were quantified in total by liquid chromatography (Agilent Technologies 1290 Infinity UHPLC chromatography, Palo Alto, USA), followed by electrospray ionization (ESI) MS-MS (Agilent 6460 mass spectrometer, Palo Alto, USA). UHPLC-ESI-MS/MS data were acquired and processed by MassHunter Qualitative Analysis B07 and MassHunter Quantitative Analysis B07 software (Agilent, USA) [65,66]. The LC-MS/MS method was previously developed and validated by our research group [14, 15]. Chromatography and MS analysis; UHPLC separation was performed using an Agilent 1290 Infinity system with an autosampler (G4226A), sampler thermostat

(G1330B), quad pump (G4204A, 1200 bar), and thermostated column compartment (G1316A). A Zorbax SB-C18 column (4.6 × 100 mm, 3.5 µm, USA) was used with a mobile phase of water (0.1% formic acid) and acetonitrile (0.1% formic acid) under a controlled column temperature of 30 °C. For MS analysis, multiple reaction monitoring (MRM) mode was used to quantify organic acids (OAs) and phenolics (PCs), optimizing precursor-to-fragment ion transitions. The scan range was 50–1300 m/z, with optimized collision energies. MS conditions included a drying gas (N_2) at 350 °C (12 L/min), nebulizing gas (N_2) at 55 psi, sheath gas at 250 °C (5 L/min), and a capillary voltage of 3.5 kV. Data acquisition was performed using the Agilent MassHunter Workstation [46,47].

2.4. Enzyme Inhibition Properties of Daisy Extracts

For the enzyme inhibition analysis, a 100 U/mg protein-containing enzyme solution, except for in-house purified hCA I and II enzymes for each enzyme, was prepared in buffer solutions detailed in reaction conditions [67]. Each inhibition study started with lower concentrations of the extract samples, and the dose was subsequently increased until more of the enzyme activity was inhibited. The samples' IC_{50} values were calculated using the GraphPad Prism 8.4.0 non-linear regression-[Inhibitor]-normalized response (*y* values 100 down to 0) model.

2.4.1. Determination of α-Amylase Inhibition

The inhibition effect of daisy extracts over α-amylase was determined by adding gradually increasing concentrations of extracts into the enzyme activity assay [68]. α-Amylase enzyme activity was measured by aiding the formation of the starch-iodine complex, and a decrease in delta absorbance of the complex at 593 nm, where the complex shows maximum absorbance via extract addition, was recorded and evaluated as a loss in enzyme activity [69]. The assay reaction consisted of 0.5 mL of the extracts (in sodium phosphate buffer 0.1 M, pH 6.9), 1 mL of 4% starch, and 0.5 mL of enzyme solutions (1 mg/mL, 5 U per reaction). After 30 minutes of the reaction at 30 °C, 0.5 mL of 1 M HCl was added to stop enzyme activity, followed by 1 mL of iodine reagent (2.5 mM iodine in 5 mM KI). A sample that did not contain starch was also set up as a blank; a control reaction was set up without enzyme, and Acarbose was used as a reference inhibitor of the α-amylase enzyme. Right after the addition of HCl, the absorbance of all the samples was measured at 593 nm [70].

2.4.2. Determination of AChE and BChE Inhibition

Daisy extracts at varying concentrations were added to a mixture containing an equal 10 mM DTNB and 10 mM substrate in 1 mL of the reaction solution. The substrate was acetylthiocholine iodide for AChE and butyrylthiocholine iodide for BChE. Right after enzyme addition (10 µL), the absorbance at 412 nm of the mixture was measured for five minutes at minute intervals [71]. Control reactions and blank reactions were set up without inhibitors and enzymes. Positive control for the AChE and BChE inhibition test was donepezil.

2.4.3. Determination of hCA I and II Inhibition

hCA enzymes were purified by Sepharose-4 B-L-Tyrosine-sulfanilamide affinity column and characterized by SDS-PAGE as previously applied [72]. The esterase activity of the CA enzyme yields a yellow-colored aromatic p-nitrophenolate compound pH of around 7.5. By taking advantage of this transformation, the esterase activity of CA enzymes can be determined spectrophotometrically [73]. Various concentrations of the daisy samples in Tris-SO_4, (0.05 M, pH 7.4), 0.07 mM of p-nitrophenyl acetate (in 1:25 acetone: water), and 20 µL of the enzyme were gently vortexed, and as soon the as the addition of the enzyme, the absorbance change at 348 nm was monitored by measuring minute intervals for three minutes. Control reactions and blank reactions were set up without inhibitors and enzymes. These steps were repeated until more than half of the enzyme activity was inhibited [74].

2.4.4. Docking Studies

The X-ray crystallography structures of AChE (PDB ID: 4EY7), BChE (PDB ID: 6T9P), α-glycosidase (PDB ID: 5NN8), α-amylase (PDB ID: 2QV4), CA (II) (PDB ID: 3HS4) and CA (I) (PDB ID: 1AZM) were downloaded from the "Protein Data Bank" website (PDB) with resolutions of 2.35 Å, 2.70 Å, 2.45 Å, 1.97 Å, 2.70 Å and 2.00 Å, respectively [75]. To investigate the binding affinities in the extracts at the active sites of the target enzymes of the major compounds detected, the 3D version of the chemical structures of the ligands was downloaded from PubChem [76]. Enzyme and ligand structures were optimized and loaded with AutoDock-Tools (v1.5.7, The Scripps Research Institute, San Diego, CA). Then, the molecular docking protocol was applied to the determined targets using AutoDock Vina 1.1.2 [77]. The best docking scores and binding interactions are analyzed by BIOVIA Discovery Studio (Accelrys Software Inc., San Diego, CA, USA, 2012).

3. Results and Discussion

3.1. Phytochemistry of Daisy Samples

Phytochemical constituents of the daisy samples were determined through LC-MS/MS chromatographic analysis against thirty-five phenolic compounds. The chromatograms of *M. chamomilla* and *A. pseudocotula* were quite similar, not surprisingly, sixteen different compounds in *M. chamomilla* and *A. pseudocotula*, and thirteen different compounds in *C. officinalis* were identified in the peak analysis. Eight of the compounds were detected in each of the three extracts: quinic acid, fumaric acid, gallic acid, chlorogenic acid, vanillic acid, quercetin, apigenin, and isorhamnetin. These were further characterized through a standard chromatogram. Only caffeic and ferulic acids were additional constituents of *M. chamomilla* from *A. pseudocotula*, while epigallocatechin gallate and taxifolin were the differentiating compounds of *A. pseudocotula* from *M. chamomilla*. Epicatechin and vitexin were detected only in *C. officinalis* extracts. The major compound of the extracts is chlorogenic acid, with a concentration of 23034.2 ng/mL in *M. chamomilla*, apigenin with a concentration of 15339.0 ng/mL for *A. pseudocotula*, and chlorogenic acid with a concentration of 8786.6 ng/mL in *C. officinalis.* Higher concentrations of vanillic acid and apigenin were detected in *M. chamomilla* and *A. pseudocotula samples.* Sibul et al. reported the study conducted to identify phenolics of the Asteraceae family plants, including the *M. chamomilla*. They reported apigenin as the major compound of *M. chamomilla* methanolic extract with 0.7 mg/g. Besides, apigenin derivatives were regarded as active biomarkers of chamomile flowers. Additionally, O-hexosyl ferulic acid, myricetin-3-O-hexoside, luteolin-O-deoxyhexosylhexoside, and rhamnetin/isorhamnetin-O-acetylhexoside were the exceptional metabolites of *M. chamomilla* [78]. Based on the results obtained from another study, 31 phenolic compounds in *A. tinctoria* var. *tinctoria* have been characterized, with hesperidin determined to be the major compound with a concentration of 22.3 mg/g extract. Caffeic acid and protocatechuic acid were other major components with 12.6 mg/g and 11.1 mg/g, respectively. It was well-known that caffeic acid had antioxidant and antiradical properties. Following these components, rutin, isoquercitrin, and cynaroside were the other major flavonoid components of *A. tinctoria* var. *tinctoria* after hesperidin among the standard compounds tested, with the amounts of 8.2 mg/g extract, 7.9 mg/g extract, and 2.9 mg/g extract, respectively. Contrary to our findings, chlorogenic acid, vanillic acid, or apigenin were not among the abundant compounds but were still detected in the extracts even in very low concentrations [79]. Gunes et al. investigated the phytochemicals of *C. officinalis.* They reported that different extracts may constitute different compounds with varying concentrations, regardless of the extraction method, hesperidin, quinic acid, fumaric acid, protocatechuic acid, protocatechuic aldehyde, chlorogenic acid, caffeic acid, salicylic acid, miquelianin, isoquercitrin, rutin, astragalin, nicotiflorin, quercetin, and luteolin was determined in the *C. officinalis* extracts. The differences between the findings may be the result of standard phytochemicals tested, nevertheless, chlorogenic acid and quinic acid were concluded to be the most abundant metabolites [24].

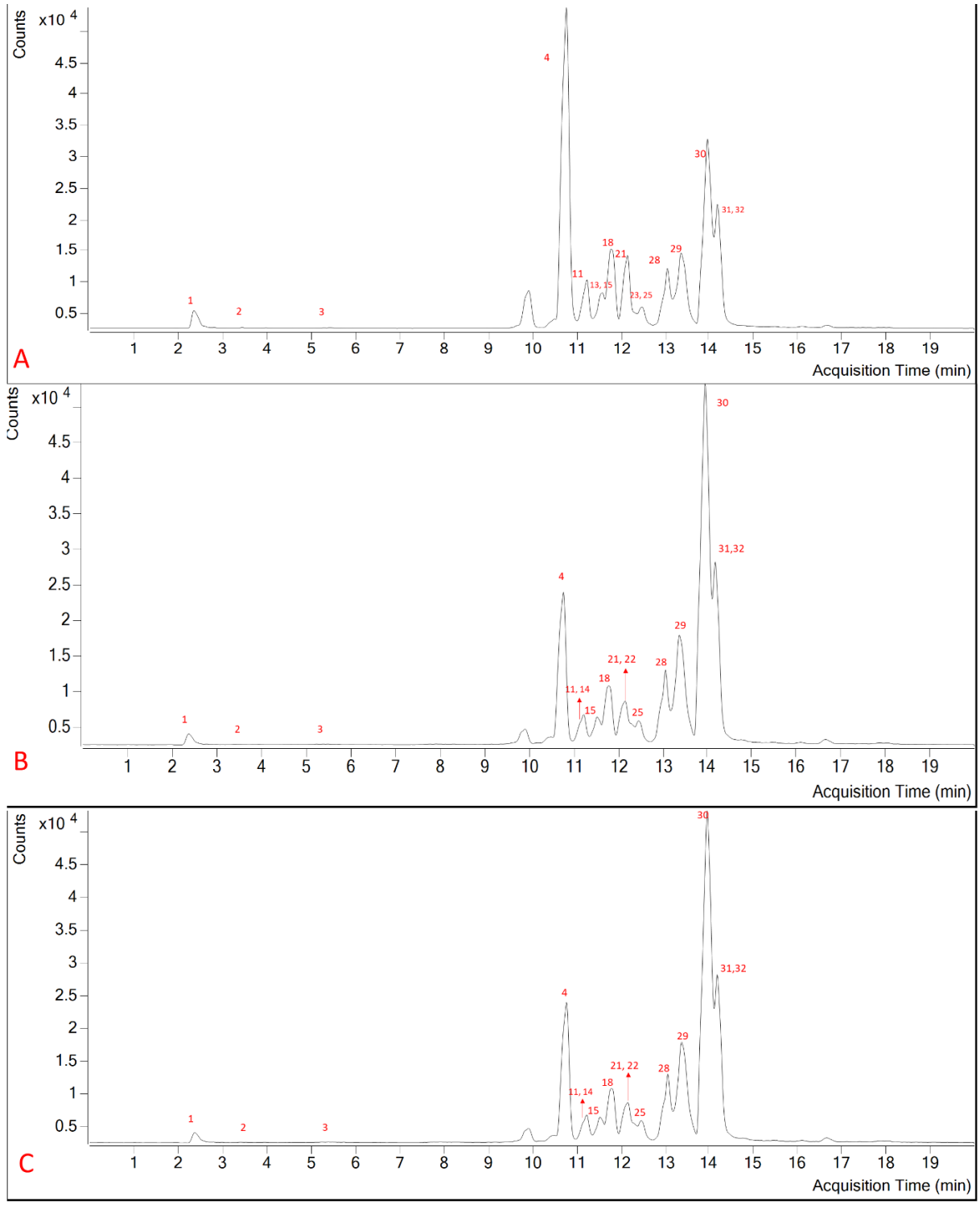

Figure 1. LC-MS/MS Chromatogram of **A**) *M. chamomilla*, **B**) *A. pseudocotula*, **C**) *C. officinalis*,

It can be concluded from quantitative LC-MS/MS results that each of the daisy species is rich in phenolic acids, which are synthesized through the shikimate pathway by deriving from gallic acid or aromatic amino acids to chlorogenic acid, fumaric acid, caffeic acid, vanillic acid, and 4-OH-benzoic acid. Phenolic acids play a major protective role in oxidative stress conditions and their individual biological properties [80].

These antioxidant properties enable the plants to play a protective role in degenerative diseases such as cardiovascular disease, cancer, diabetes, inflammation, and many more [81]. In addition to this, specific flavonoids such as vitexin and taxifolin, whose remarkable biological properties have been investigated for their role in diabetes in recent studies, were determined. Especially, it was proven that taxifolin demonstrated antioxidant and enzyme inhibition properties [82,83]. Also, it has gained attention due to its significant role in nervous system-related diseases such as AD and Parkinson's disease (PD). Naringin is another versatile molecule that has several benefits to the body regarding its antioxidant [84,85]. It has a protective effect on experimental hindlimb ischemia-reperfusion injury in rats, and so on [86]. Studies reported the role of apigenin in neuroinflammation across different pathologies: neurodegenerative diseases such as multiple sclerosis, PD, AD, cancer, cardiovascular diseases, and cognitive and memory disorders. Evidence suggests that apigenin modulates various signaling pathways involved in inflammation, oxidative stress, and cell death, offering neuroprotective effects in experimental models [87].

Table 1. Quantitative LC-MS/MS analysis results of daisy samples

No	Compounds	***M. chamomilla***		***A. pseudocotula***		***C. officinalis***	
		Conc. (ng/mg of sample)	RT (min)	Concentration (ng/mg of sample)	RT (min)	Conc. (ng/mg of sample)	RT (min)
1	Quinic acid	8488.0	2.4	4401.4	2.4	1141.0	2.4
2	Fumaric acid	498.2	3.7	95.8	4.0	917.0	4.0
3	Gallic acid	49.0	5.3	60.0	5.2	32.7	5.3
4	Chlorogenic acid	23034.2	10.8	9030.6	10.8	8786.6	10.8
5	Pyrogallol	-	-	-	-	-	-
6	Cyanidin-3-*O*-glucoside	-	-	-	-	-	-
7	Keracyanin chloride	-	-	-	-	-	-
8	Chlorogenic acid	-	-	-	-	-	-
9	Peonidin-3-*O*-glucoside	-	-	-	-	-	-
10	Catechin	-	-	-	-	-	-
11	4-OH-benzoic acid	220.9	11.3	549.8	11.3	-	
12	Epicatechin	-	-	-		72.5	11.2
13	Caffeic acid	346.9	11.5	-		-	
14	Epigallocatechin gallate	-	-	265.4	11.5	371.7	11.2
15	Vanillic acid	3388.8	11.6	9428.0	11.6	625.2	11.6
16	Syringic acid	-	-	-	-	-	-
17	Vitexin	-	-	-	-	442.1	11.7
18	Naringin	104.7	11.9	166.9	11.9	-	-
19	Hesperidin	-	-	-	-	-	-
20	Ellagic acid	-	-	-	-	-	-
21	*p*-Coumaric acid	136.7	12.3	196.7	12.3	-	
22	Taxifolin	-		186.0	12.4	54.4	12.4
23	Ferulic acid	110.8	12.5	-	-	116.0	12.5
24	Vanillin	-	-	-	-	-	-
25	Rosmarinic acid	54.4	12.5	449.3	12.6	-	
26	Myricetin	-	-	-	-	-	-
27	Resveratrol	-	-	-	-	-	-
28	Luteolin	690.1	13.4	759.0	13.4	-	
29	Quercetin	724.3	13.5	936.3	13.5	214.3	13.5
30	Apigenin	5471.0	14.0	15338.9	14.0	13.5	13.9
31	Naringenin	11.8	14.0	32.6	14.0	-	-
32	Isorhamnetin	800.0	14.2	1237.1	14.3	248.9	14.2
33	Curcumin	-	-	-	-	-	-
34	Chrysin	-	-	-	-	-	-
35	Galangin	-	-	-	-	-	-

RT: Retention Time

3.2.3.2. Molecular Docking Studies

The phytochemical content analysis results revealed that chlorogenic acid, apigenin, and vanillic acid are the major compounds in the extracts; thus, molecular docking studies were performed to estimate and determine the possible inhibition potential and mechanism of the extracts and to make a decision to investigate *in vitro* enzyme inhibition analysis. The major compounds were prepared as

ligands separately against catalytic protein targets, including AChE, BChE, α-amylase, AG, hCA I, and hCA II. The binding affinity of the chlorogenic acid-AChE complex was calculated as -10.0 Kcal/mol and was the highest docking score of the six enzymes (Table 2). The interactions of chlorogenic acid with AChE were determined as 7 H bonds with GLY: 121,122; SER: 203, 293; ARG: 269, HIS: 447. The molecule's aromatic ring has a π-π stacked interaction between the active center amino acid, TRP:86 (Figure 1). The chlorogenic acid-BChE complex was calculated as -9.0 Kcal/mol (Table 2). The interactions of the complex were determined as 5 H bonds with GLU: 197; TYR:128, 332, and ASN:83. The aromatic ring of the molecule has made a π-π stacked interaction with TYP:82. The binding affinity of the chlorogenic acid-hCA II complex was calculated as -8.2 Kcal/mol (Table 2). The interactions of chlorogenic acid with hCA II were determined as 4 H bonds with PRO:201, THR:200, and GLN:92. Also, the aromatic ring of the molecule made 1 π-π T-shaped interaction with HIS:94 and 2 π-π-alkyl interactions with VAL:143 and VAL:121, also a π-sigma interaction with LEU:198 amino acid residues. The binding affinity of the chlorogenic acid-hCA I complex was calculated as -7.5 Kcal/mol (Table 2). The interactions of chlorogenic acid with hCA I were determined as 6 hydrogen bonds with HIS:64, GLN:242, TRP:5, and ASN11. Also, the aromatic ring of the molecule made a π donor hydrogen bond with the SER:231 amino acid residue. Chlorogenic acid showed a high binding affinity with α-amylase (2QV4) and was calculated as -8.1 Kcal/mol (Table 2). The interactions of chlorogenic acid with α-amylase (2QV4) were determined as 6 hydrogen bonds with ASP:353, 356; ALA:310; ASN:301; ILE:312, and ARG:346. The chlorogenic acid-α-AG(5NN8) complex's docking score was calculated as -7.3 Kcal/mol (Table 2). 5 hydrogen bonds of -7.3 with AG's ASP:518, 282, 616 residues. The aromatic ring of the molecule made a π-π T-shaped interaction with the PHE:649 residue.

Table 2. Molecular interactions of ligands with α-amylase (A:2QV4), AChE (B: 4EY7), BChE (C:6T9P), Carbonic anhydrase II (D: 3HS4), α-glycosidase (E: 5NN8) and carbonic anhydrase I (F: 1AZM).

Complex	**Docking scores (Kcal/mol)**	**Interactions types**	**Interacting residues**
hCA I (1AZM)-Chlorogenic acid	-7.5	H bonding	GLN:242; HIS:64
hCA II (3HS4-Chlorogenic acid	-8.2	H bonding π alkyl π-π T-shaped π sigma	THR:200; GLN:92; PRO:201 VAL:121; 143 HIS:94 LEU:198
AChE (4EY7) -Chlorogenic acid	-10.0	H bonding π-π stacked π alky	GLY: 121,122; SER:203, 293; ARG:269 TRP:286 PHE:338
BChE (6T9P)-Chlorogenic acid	-9.0	H bonding π-π stacked	GLU: 197; TYR:128, 332; ASN:83 TRP:82
α-Glycosidase (5NN8)-Chlorogenic acid	-7.3	H bonding π-π T-shaped	ASP:518, 282, 616 PHE:649
α-Amylase (2QV4)-Chlorogenic acid	-8.1	H bonding	ASP:353, 356; ALA:310; ASN:301; ILE:312; ARG:346

In a study, it was reported the inhibition effect of some phenolic compounds, including chlorogenic acid, on serum AChE and both *in silico* and *in vitro* inhibition analysis. Their docking findings were highly consistent with the present docking results, and they concluded that the high inhibition effect of phenolic compounds arises from their large number of functional groups that can interact with the catalytic site of the enzyme [88]. Similarly, it was identified the phenolics in *M. chamomilla* extracts and detected compounds were docked to AChE with an *in silico* study, and they reported that kaempferol-3-O-rutinoside showed a high binding affinity with-10.21 Kcal/mol energy [23].

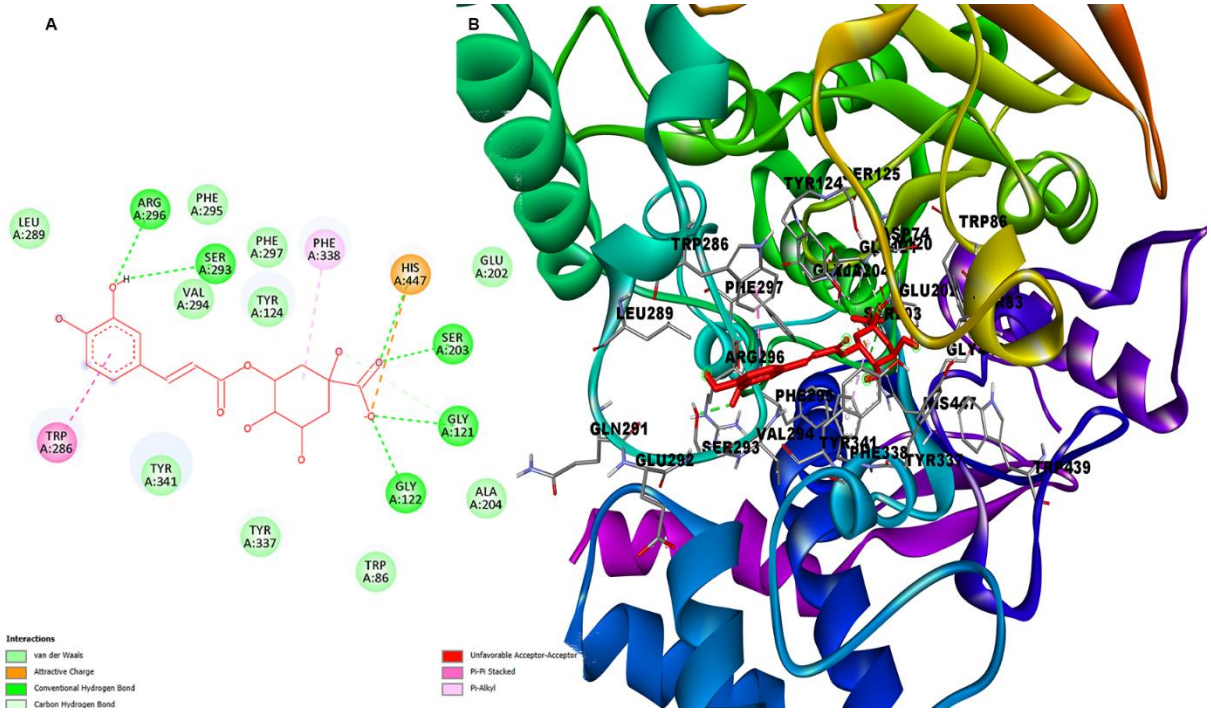

Figure 2. Docking poses of chlorogenic acid to AChE **A**; 2D and **B**; 3D

The apigenin-AChE complex was calculated as -10.4 Kcal/mol and was the highest docking score of the six enzymes (Table 2). The interactions of the complex were determined as 3 hydrogen bonds with GLY: 121; TYR:124, and SER:203. The molecule has made 3 π-π stacked interactions with TYR: 341 and TRP: 286, and a π-sigma interaction with TYR: 341 amino acid residues (Figure 3). Viswanathan and co-workers investigated the binding interactions of some phenolics, including apigenin, with AChE, and they determined that the binding affinity of apigenin to the catalytic site was -8.87 Kcal/mol, while this value was -11.03 Kcal/mol for donepezil [89]. In another study, it was reported in their study investigated the cholinesterase inhibitory activities of apigenin through molecular docking studies, and they determined the binding affinity of apigenin to BChE as 5.91 Kcal/mol. In addition, one of the hydrogen bonds was determined between the phenolic hydroxyl of the apigenin and the carbonyl group of SER198 (1.83 Å) of BChE. The second was on the hydroxyl (7-OH) of the benzopyran ring and carbonyl group (C=O) of ASP:70 (1.5 Å). π-π stacking interaction between the benzopyran benzene ring and phenyl group of TYR:332 (4.96 Å) and hydrophobic interaction with residues of ALA:199, TRP:231, PRO:285, LEU:286, VAL:288, PHE:329, TYR:332, PHE:398 was also recorded. The polar interactions were realized by SER:198 and SER:287. Lastly, negative load interaction was detected between the ASP:70 residue and apigenin [90]. The binding affinity of the vanillic acid-AChE complex was calculated as -6.8 Kcal/mol (Table 2). The interactions of vanillic acid with AChE were determined as 4 hydrogen bonds with TYR: 133; TYR: 337, GLY: 121, and SER: 203, and an π-alkyl interaction with TYR: 124. Also, the aromatic ring of the molecule made a π-π stacked interaction with TRP: 86 amino acid residues (Figure 4). According to the results, vanillic acid was the lowest affinity-bearing molecule among the three major compounds, and based on the conclusion, the $-OCH_3$ group in vanillic acid structure has a partially activity-reducing effect, so this compound may have lower bioactivity than hydroquinone [88].

3.3. Enzyme Inhibition of Daisy Samples

Daisy samples were subjected to *in vitro* enzyme inhibition analysis by simply incorporating the different concentrations of the samples into the reaction mixture. The applied dose of the extracts was increased up to half or more, and the enzyme activity of the control reaction was inhibited. Based on the normalized derivatives (percentage inhibition) of the results, IC_{50} values of daisy samples on each enzyme were calculated. According to the AChE inhibition results, each daisy extract inhibited AChE

activity with greater IC_{50} values than the reference donepezil; *A. pseudocotula* was the greatest with a 1.4 µg/mL IC_{50} value. The following order was *C. officinalis* with 3.6 µg/m, *M. chamomilla* with 4.4 µg/mL, and donepezil with 8.8 µg/mL IC_{50} values. BChE inhibition assay showed a similar order in results, except that donepezil showed the lowest IC_{50} value, while *M. chamomilla, A. pseudocotula*, and *C. officinalis* showed similar inhibitions with 7.1 µg/mL and 9.3 µg/mL. IC_{50} values, which are quite lower than the IC_{50} value of donepezil, 15.5 µg/mL. According to the AG inhibition results, *A. pseudocotula* showed higher inhibition with a 130.0 µg/mL IC_{50} value among other daisies, *M. chamomilla*, and *C. officinalis* with 155.2 µg/mL and 174.8 µg/mL IC_{50} values. Even though the samples showed AG inhibition, IC_{50} values were quite higher than the reference Acarbose, 9.4 µg/mL. This finding concluded as insufficient potential to be used as a drug candidate. In addition, α-amylase inhibition could not be determined, possibly due to the presence of β-amylases in the extracts interfering with the assay [91]. In both hCA I and hCA II inhibition assays, *A. pseudocotula* exhibited similar inhibition with 4.6 and 8.4 µg/mL IC_{50} values to the reference inhibitor, acetazolamide, which has 4.6 and 10.0 µg/mL IC_{50} values for hCA I and hCA II, respectively. Lastly, the hCA I and hCA II inhibition order was similar and as follows: *M. chamomilla* with 6.2 and 14.0 µg/mL, and *C. officinalis* with 7.8 and 14.9 µg/mL, respectively.

Table 2. Half maximal enzyme inhibition concentrations (IC_{50}, µg/mL) results for the plant extracts. *Acarbose was employed as a positive control for α-glycosidase, and donepezil for AChE and BChE. The IC_{50} value for acetazolamide, ND, was not detected.

Enzyme	***M. chamomilla***		***A. pseudocotula***		***C. officinalis***		**Reference***	
	IC_{50}	**r^2**	**IC_{50}**	**r^2**	**IC_{50}**	**r^2**	**IC_{50}**	**r^2**
AChE	4.4	0.9695	1.4	0.9849	3.6	0.9896	8.8	0.9765
BChE	29.9	0.9458	7.1	0.9976	9.3	0.9629	15.5	0.9743
α-Glycosidase	155.2	0.9537	130.0	0.9812	174.8	0.9158	9.4	0.9874
α-Amylase	ND	ND	ND	ND	ND	ND	ND	ND
hCA I	6.2	0.9216	4.6	0.9926	7.8	0.9578	4.6	0.9916
hCA II	14.0	0.9962	8.4	0.9893	14.9	0.9934	10.0	0.9921

Here, each of the daisy species was determined to be effective in inhibiting both AChE and BChE. Besides, there were IC_{50} values detected lower than that of donepezil, which could be a sign of a potential drug candidate with less adverse effect, as the dose required to be applied will be lower. Recent studies performed with *M. chamomilla* have been mostly focused on animal experiments to investigate the effect of extracts on neurological ailments such as motor coordination, memory impairment, and hippocampal neuron damage [92]. No specific study investigating the anticholinergic activity of *M. chamomilla* extracts has been published so far. It was reported that *A. stiparum* subsp. *sabulicola* methanolic extracts inhibited AChE and BChE with 490.5 µg/mL and 142.1 µg/mL IC_{50} values [93]. Also, it was determined that the AChE and BChE inhibition of *C. officinalis* oils with 1.14 ± 0.04 mg/mL and 1.2 ± 0.06 mg/mL, while Galantamine with 0.003 ± 0.0001 mg/mL and 0.01 ± 0.001 mg/mL, IC_{50} values for AChE and BChE, respectively [22].

Qasem et al. have studied the antidiabetic properties of *M. chamomilla* oils, and they have found that *M. chamomilla* oils inhibited the α-amylase and α-glycosidase with IC_{50} values of 121.44 ± 0.05 and 265.6±0.03 µg/mL, while these values were found as 396.4 ± 5.16 and 199.5 ± 1.12 µg/mL for Acarbose. Even though the α-glycosidase inhibition values of the report and the study are similar, our findings were quite weak against Acarbose [94]. To the best of our search, there is no report on an anti-diabetic potential investigation through α-amylase and α-glycosidase inhibition of *Anthemis* sp. It was reported that α-amylase and α-glycosidase inhibition of *C. officinalis* flowers with 3.1 ± 0.16 mg/mL and 1.1 ± 0.01 mg /mL, while Acarbose inhibition was 0.8 ± 0.03 and 0.9 ± 0.07 mg/mL, respectively [22]. Different species from the Asteraceae family, such as *H. plicatum, A. absinthium,* and *A. schischkinii* have been tested for their potential to inhibit hCA I and hCA II enzymes, these studies reported inhibition of the species with great potential [95]. So, the inhibition effects of plants and plant-derived compounds on enzymes had great importance [96]. However, no study was available that determined the inhibition potential against CAs of the genera tested in this study.

4. Conclusion

Enzyme inhibition is the golden strategy of both traditional and modern pharmacology, as the modulation of metabolism enables the fixing of abnormalities. Currently, available drugs on the market to treat various illnesses and ailments, from cancer to infections, are enzyme inhibition-based strategies that also encompass the future white hope. The difference between traditional and modern pharmacology is the "wonder" drug strategy that aims to be beneficial from more than one perspective. This is quite possible with plants that are highly rich in their phytochemicals, with various biological activities. There are hurdles with plants to overcome, such as a lack of standardization, proper definition of their effectiveness, quality, and toxicology. The present study successfully identified the plant metabolites of three daisy species, *M. chamomilla*, *A. pseudocotula*, and *C. offinalis*, and detected highly valuable phenolics and flavonoids such as quinic acid, caffeic acid, vanillic acid, and apigenin at high concentrations and many other metabolites. Their rich content was evaluated by computer-based enzyme inhibition analysis for their potential to inhibit the enzymes that are associated with health-threatening neurological and metabolic diseases such as AD, PD, diabetes, and glaucoma. Docking results in low cost and less effort, providing insight to perform *in vitro* enzyme inhibition analysis, whose results were quite consistent with *in silico* analysis. The overall results showed that *M. chamomilla*, *A. pseudocotula*, and *C. offinalis* plants inherit the property of the Asteraceae family and have the potency to be a "wonder drug" candidate.

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