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Determination of secondary metabolites of *Cydonia oblonga* (Quince) by LC-MS/MS method together with evaluation of its antioxidant and cholinergic potentials

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Abstract: In this study, quantification of secondary metabolites of Cydonia oblonga (Quince) leaves by LC-MS/MS and antioxidant, cholinergic and α-Glycosidase activities were reported. LC-MS/MS analyses of the studied extract showed that the highest phenolic constituents of species were catechin (2024.9 ± 80.9 µg/L), vanillic acid (1121.8 ± 39.3 µg/L) and fumaric acid (486.7 ± 16.5 µg/L). The ethanol extract of quince leaves inhibited the α-glycosidase enzyme with an IC50: 0.6 µg/mL value. In order to determine the anti-Alzheimer activity of the plant extract, inhibition studies were carried out on acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes. The IC50 values of the plant extract were found to be 1.3 µg/mL for AChE and 1.5 µg/mL for BChE. In addition, free radical scavenging and metal reduction tests were performed to determine the antioxidant activity of the plant and the results were compared with Trolox. It was understood that the antioxidant potential of the plant was lower than Trolox. The results from biological abilities, molecular docking of catechin hydrate as abundant phenolic compounds in quince leaves extract to be an inhibitor candidate for AChE, BChE and α-glycosidase.

Keywords: Quince; enzyme inhibition; antioxidant; Alzheimer's disease; molecular docking; polyphenol content. © 2024 ACG Publications. All rights reserved.

1. Introduction

Plants have active ingredients according to their species and their habitats. These ingredients can help humanity for biological activities [1-3]. There are millions of plant seeds in nature, each of them has their unique structure which causes a great biodiversity [4,5]. Alkaloids, flavonoids, terpenoids, and other phytochemicals found in nature can be used in the treatment of many diseases [6,7]. Many medicines are inspired by the molecules from nature. For example, painkillers such as

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aspirin, antimalarials such as quinine, and heart medicines such as digoxin are derived from plant sources [8].

Since ancient times, plants have been used to treat disease [9]. Many civilizations have used plants for treatments. The crucial examples are Traditional Chinese Medicine, Indian Ayurveda, and indigenous medical practices. This knowledge has inspired modern pharmaceutical research and the medicines nowadays[10]. Today, many people choose to support their health by using herbal supplements. For example, *Ginkgo biloba*, echinacea, and herbal extracts containing various vitamins and minerals are popular [11]. Modern research is working intensively to understand the effects of plants on health and to develop new treatments [12,13]. There is still ongoing research about the potential use of plant-derived compounds in the treatment of cancer, infections, and other diseases [14,15].

Although many studies have been conducted on the pharmaceutical properties of plants so far, studies on the leaves of fruit trees have accelerated recently [16-18]. The leaves of fruit trees are rich in powerful antioxidant compounds such as flavonoids, polyphenols, and tannins [19]. These compounds prevent cell damage by reducing oxidative stress caused by free radicals in the body and may reduce the risk of various chronic diseases [20,21]. The leaves of some fruit trees contain compounds that reduce inflammation [22]. These leaves may help treat inflammatory diseases (such as arthritis, asthma, and inflammatory bowel disease). For example, olive tree leaves (*Olea europaea*) are known for such anti-inflammatory properties [23]. Extracts from the leaves of fruit trees are effective against various bacteria and viruses. These leaves can be used to treat conditions such as skin infections and respiratory diseases [24,25].

Quince tree (*Cydonia oblonga*) is a fruit tree belonging to the *Rosaceae* family [26]. The leaves of the quince tree are oval-shaped, bright green and hairy. The leaves turn yellow and fall off in autumn. The white or pale pink flowers usually bloom in spring, in April and May. The flowers are usually found singly or in small groups and emit a pleasant scent. Quince fruit is large, has a hard and hairy peel and ripens in yellow. The inside of the fruit is hard, granular and slightly acidic [27]. Quince fruit is consumed cooked or processed rather than raw. It is used in many delicious recipes such as quince jam, marmalade, compote and quince dessert. Quince is also used in jelly making due to its high pectin content. In addition, quince juice, liqueur or wine is made in some regions. Quince is beneficial for health with its high vitamin C, fiber and antioxidant content. It regulates digestion, strengthens the immune system and has anti-inflammatory properties [28].

Alzheimer's (AD)is the most common type of dementia worldwide and is a neurodegenerative disease that is increasing nowadays. [29-31]. The disease starts with the loss of brain cells, which eventually causes brain tissue to shrink. This causes symptoms such as memory loss, decreased ability to think and reason, and changes in behavior [32,33]. AD usually occurs in people aged 65 and older, but it can begin at an earlier age [34-36]. The exact cause of AD is not known, but it is assumed that some factors play a role in the development of the disease [37]. The risk increases in people with a family history of AD [38,39]. In particular, it has been reported that people who carry the APOE-e4 gene are at higher risk of developing the disease. Biochemical changes in the brain are another reason to explain the disease. People with AD have abnormal protein deposits in the brain, such as amyloid plaques and tau tangles. These deposits disrupt the ability of brain cells to communicate with each other [40]. The biggest risk factor for AD is aging. The risk of developing the disease increases with age [41].

The relationship between AD and cholinesterases is important in terms of the mechanism of action of drugs used in the treatment of the disease [42,43]. In AD, the levels of some neurotransmitters that allow nerve cells in the brain to communicate with each other are reduced [44]. One of these neurotransmitters is acetylcholine. Acetylcholine is a neurotransmitter that plays a critical role in the transmission of messages between nerve cells. In particular, it is vital for learning and memory functions [45]. In AD, decreased acetylcholine levels contribute to memory loss and cognitive dysfunction, one of the main symptoms of the disease. Cholinesterases are enzymes that rapidly break down acetylcholine in synapses, rendering it inactive. AChE breaks down acetylcholine in the synapse gap into acetate and choline, thus terminating neurotransmission. The activity of these enzymes helps

regulate acetylcholine levels under normal conditions [46,47]. In AD, acetylcholine levels in the brain are significantly reduced. This reduction leads to poor neurotransmission across cholinergic synapses, which in turn leads to impaired cognitive function. One of the medications used to manage this condition is cholinesterase inhibitors [48]. Cholinesterase inhibitors block the cholinesterase enzymes that break down acetylcholine and make it inactive [49]. This inhibition causes more acetylcholine to accumulate in the synapses. As a result, communication between nerve cells is temporarily improved, leading to temporary improvement in cognitive function in patients [50].

Diabetes is a long-term condition where the body struggles to control blood sugar levels [51]. By permitting glucose, an essential energy source, to enter the cells, the hormone insulin aids in the production of energy by the cells [52]. Either insufficient insulin synthesis, inappropriate insulin use, or both can lead to diabetes. The outcome is hyperglycemia, or elevated blood sugar, which can lead to chronic health problems [53].

The role of insulin and glucose metabolism makes enzymes and diabetes related. Enzymes, which are proteins that catalysts chemical reactions in the body, are essential for the conversion of glucose into energy and the preservation of blood sugar balance [56]. It is believed that a major factor in the onset and progression of diabetes is enzyme malfunction.

Diabetes is treated with α-glycosidase inhibitors (AGIs), which lowers blood glucose spikes after meals by blocking the action of these enzymes. By delaying the conversion of carbohydrates to glucose, these drugs restrict the amount of glucose that can be absorbed from the small intestine. As a result, blood glucose levels rise more gradually and under more regulated circumstances [57].

This study evaluated the biological activity of quince (Cydonia oblonga) leaves. Several tests of the leaves' biological activity were conducted after they were extracted in ethanol. The polyphenol content of the generated extract was determined using the LC/MS-MS method. Enzyme inhibition experiments targeting AChE and BChE were utilized to assess potential anti-AD capacity, whereas α-glycosidase inhibition assays were employed to assess antidiabetic potential. Moreover, antioxidant activity was investigated.

2. Experimental

2.1. Chemicals

All chemicals used in experimental studies were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Since the uncertainties arising from purity are very low, the purity values of the compounds specified in Table 2 are not given individually, and the purity values of the compounds were in the range of 96-99% purity.

2.2. Preparation of Extract of Quince Leaves (EQL)

The plant sample was purchased from a local market in Aksaray/Turkey and species identification was performed (Herbarium number: EBYU-Korkmaz:4656). The quince leaf samples were dried in cool and shaded conditions. Dried leaves were ground until they became powder. 20 g of this powdered sample was weighed and 400 mL of ethanol was added to it. This prepared solution was stirred for 24 hours in the dark with the help of a magnetic stirrer (ARE Magnetic Stirrer, VELP Scientifica, Italy). At the end of this period, the ethanolic extract was filtered through filter paper (Whatman No. 1, Sigma Aldrich, Germany) and the pulp was removed. Then, ethanol was removed from the medium with the help of a rotary evaporator (Heidolph Hei-Vap Series, Heidolph Instruments, Nuremberg, Germany). After removing the solvent, the obtained extract was stored at -20°C until further studies [58].

2.3. Determination of Total Phenolics

Total phenolic content of EQL was determined according to the Folin-Ciocalteu. First, EQL was prepared stock solution at a concentration (1 mg/mL). Then, 1 mL of this stock solution was

transferred to a test tube and the volume was adjusted to 25 mL with distilled water. Then, 0.5 mL Folin-Ciocalteu reagent and 1.5 mL sodium carbonate (2%) were added. Samples were vortexed and incubated at room temperature for 30 min, then absorbance was measured at 760 nm. Distilled water was used as blank and control. A calibration curve of gallic acid was prepared and the total phenolic content of EQL was determined from the linear regression equation of this curve. Results were given as gallic acid equivalents per mg [59].

2.3. Determination of Total Flavonoids

Flavonoids, which originate from plants, are the group of polyphenolic compounds that are most abundant in the human diet and have antioxidant activity. Total flavonoid content in EQL was determined according to the procedure of Gulcin et al. [60]. To determine total flavonoids in EQL, the mixture containing 1 mL of EQL, 0.1 mL of aluminum nitrate (10%), and 0.1 mL of aqueous potassium acetate (1.0 M) was diluted with 4.3 mL of ethanol. After the solution was incubated at room temperature for 30 minutes, its absorbance was measured spectrophotometrically at 415 nm. Distilled water was used as blank and control. A calibration curve of Quercetin was prepared for the standard chart, and the flavonoid contents were determined from the linear regression equation of the calibration curve. Results were given as quercetin equivalent per mL of oil [61].

2.4. Phenolic Composition Using LC-MS/MS Analysis

The phenolic content of Quince Leaf (QL) was determined using a LC-MS/MS (Nexera model Shimadzu UHPLC) device with a triple quadrupole mass analyzer. A C18 Inertsil ODS-4 (3.0 mm x 100 mm, 2 µ) analytical HPLC column was used in a 40 °C column oven. A gradient of Water, 0.1 % formic acid (Mobile Phase A) and Methanol, 0.1 % formic acid (Mobile Phase B) were used with 0.5 mL/min flow rate. Injection volume of each extract was used as 4 µL [62]. Measurements were performed using the multiple reaction tracking (MRM) mode. Experiments were performed in triplicate; the average was taken and the results were presented quantitatively (Table 2).

2.5. Antioxidant Activity

2.5.1. Reducing Capability

The CUPRAC method using copper ions and a heterocyclic organic compound neocuproine (2,9-dimethyl-1,10-phenanthroline) and FRAP methods were applied [63-65] for evaluation of reducing capability of the quince leaves. The stock solution of EQL and Trolox were mixed with the reagent in a test tube. The reaction mixture was incubated in the dark for 30 minutes to allow the antioxidants to reduce Cu2+ ions to Cu+. The absorbance of the reaction mixtures was measured at (450 nm). The intensity of the color formed is proportional to the reduced capacity of the antioxidants present in the sample.

The Ferric Reducing Antioxidant Power (FRAP) method, which also uses iron ions was performed [64]. FRAP assay is a common method used to measure the antioxidant activity of various compounds, including plant extracts like EQL. This assay measures the ability of an antioxidant to reduce a ferric ion (Fe3+) to a ferrous ion (Fe2+) in the presence of a reducing agent. The reduction of the ferric ion is accompanied by the formation of a colored ferrous-tripyridyltriazine (TPTZ) complex, which can be measured spectrophotometrically [65].

2.5.2. Radical Scavenging Activities

Two different methods were used to determine the radical scavenging activity of EQL compared to Trolox. The DPPH (1,1-diphenyl-2-picrylhydrazyl) assay is widely used to evaluate the antioxidant capacity of various compounds, including plant extracts and essential oils. This assay was

realized according to the method of Blois [66] as described previously [67]. It measures the ability of an antioxidant to donate an electron or hydrogen atom to the stable DPPH radical, thereby reducing it to a non-radical form. Small amounts of EQL solutions were mixed with the DPPH solution. The mixture was allowed to incubate in the dark at room temperature for a specified period of time. During this incubation period, the EQL's antioxidants react with the DPPH radical, causing the purple color of the DPPH solution to fade. After the incubation period, the decrease in absorbance (color intensity) of the DPPH solution was measured at 517 nm [68]. A lower absorbance indicates a higher radical scavenging activity of the EQL.

Gulcin's method was used to determine the ABTS radical scavenging ability of EQL [69]. In this assay, an aqueous solution of 7.0 mM ABTS was reacted with 2.5 mM $K_2S_2O_8$ to produce a radical cation ($ABTS^{\cdot+}$). The produced ($ABTS^{\cdot+}$) cation was diluted with phosphate buffer (0.1 M, pH 7.4) and the absorbance value of the control was adjusted to a constant value at 734 nm. Then, 1 mL $ABTS^{+\cdot}$ solution was added to 3 mL of EQL and Trolox, at different concentrations and incubated for 30 min. The residual absorbance of $ABTS^{\cdot+\cdot}$was measured at 734 nm [70]. The radical scavenging activity, expressed as the percentage inhibition of DPPH and ABTS radicals, was calculated using the following formula:

Radical Scavenging Activity (%) = (1− Absorbance of sample / Absorbance of control) x 100

The data from the experiment was used to plot a graph of percentage inhibition (radical scavenging activity) against the concentration of EQL and other antioxidant standards. The concentration at which the samples scavenge 50% of the DPPH and ABTS radicals is known as the IC_{50} value. The lower IC_{50} value the higher antioxidant activity. These assays provide valuable information about the antioxidant potential of EQL and its ability to neutralize free radicals, which are associated with oxidative stress and various health issues [71].

2.6. Cholinesterase Enzymes Inhibition Assays

The inhibition effects of the samples on AChE/BChE enzymes were tested using the Ellman's method [72]. Stock solutions of each sample were prepared by dissolving them in DMSO to a final concentration of 1 mg/mL. These stock solutions were diluted with deionized water to obtain samples at different concentrations. For the anticholinesterase activity assays, the reaction mixture in the sample cuvette was prepared by sequentially adding 100 µL of buffer (1 M, pH 7.8 phosphate buffer), 10 µL of enzyme (0.385 U/mL for BChE and 0.880 U/mL for AChE), 10 µL of the EQL at different concentrations, 50 µL of DTNB (10 mM), and 50 µL of substrate solution (10 mM acetylthiocholine iodide / 0.5 mM butyrylthiocholine iodide). The reaction was initiated, and the amount of enzymatic hydrolysis of both substrates was determined by measuring the absorbance of the yellow 5-thio-2-nitrobenzoate anion, formed by the reaction of DTNB with thiocholine, at a wavelength of 412 nm. Tacrine was used as a positive control IC_{50} values were calculated by plotting the percentage of activity against concentration [73].

The α-glycosidase enzyme inhibition experiments for EQL were conducted with slight modifications to the method which were previously reported in the literature [74,75]. In this procedure, 10-100 µL of the sample, whose inhibitory effect was to be assessed, was added to a test tube containing 200 µL of phosphate buffer (0.1 M, pH 7.4) and 20 µL of enzyme solution (0.11 U/mL in phosphate buffer, pH 7.4). The mixture was pre-incubated at 35°C for 10 minutes. Following the incubation, 20 µL of p-nitrophenyl-α-D-glucopyranoside (5 mM in phosphate buffer, pH 7.4) was added, and the mixture was incubated again at 35 °C. The absorbance was measured spectrophotometrically at 405 nm. The results were reported as the IC_{50}, representing the sample concentration that inhibited 50% of the enzyme activity. Acarbose was used as the positive control. IC_{50} values for all enzymes were calculated by means of the graphs obtained by making activity measurements at different concentrations for both EQL and standard inhibitors [76-77].

2.7. Statistical Analyses

Statistical analyses were performed using Student's t-test (GraphPad Prism 6, GraphPad, La Jolla, CA, USA, Software 7.0). The data are presented as means ± standard deviations (SD). The minimum significance level was set at $p < 0.05$. All experiments were conducted in triplicates.

3. Results and discussion

3.1. Total Phenolics and Flavonoids Contents

Phenolic compounds are a group of secondary metabolites that are widely found in plants and are of great importance in terms of biological activities [78]. They structurally contain aromatic rings containing one or more hydroxyl groups. They serve in the defense mechanism of plants, increasing their resistance to environmental stress and also attract attention with their antioxidant, antimicrobial, anti-inflammatory and anticancer activities [79]. The beneficial effects of phenolics on human health have been associated with their capacity to prevent cellular damage caused by free radicals [80,81]. The total phenolic content of EQL was determined with the Folin-Ciocalteu method as previously mentioned. Gallic acid equivalent from the linear regression equation was obtained from different gallic acid concentrations. The total phenolic content in one milliliter of EQL was determined to be 188.63±0.015 µg GAE/mL. According to this result, it is possible to say that the ethanol extract of quince leaves is a good phenolic source. Because when EQL is compared with ethanol extracts of some plants such as ginger (137.5 µg GAE/mL) and thyme (158.0 µg GAE/mL), it is understood that the total phenolic content of EQL is higher [82,83].

Flavonoids, an important subclass of this group, provide color formation in plants (especially flowers and fruits) and also contribute to the protection of plants against UV rays [84]. The structure of flavonoids is characterized by a benzene ring based on the C6-C3-C6 skeleton, and this structure allows them to exhibit strong antioxidant properties [85]. Flavonoids are considered bioactive compounds that provide protective effects against cardiovascular diseases, cancer, diabetes and neurodegenerative diseases in terms of health [86]. Determination of total phenolic and flavonoid content is a fundamental step to evaluate the potential health benefits of a plant extract and to obtain a biological activity profile [87]. These parameters are directly related to the biological activities of the plant, such as antioxidant capacity, antimicrobial activity and anti-inflammatory properties. Therefore, the determination of the amounts of these compounds is of great importance to reveal the therapeutic potential of plant extracts and to evaluate them in terms of bioavailability [88]. Quercetin, a flavonoid molecule that we used as a reference in our study, is a well-known polyphenol of plant origin with anti-inflammatory and antioxidant properties. Flavonoids could chelate metals by forming complexes with metal ions [89]. The total flavonoid content in EQL was calculated according to the aluminum nitrate method. For the determination of total flavonoids in EQL, a quercetin equivalent from the linear regression equation was obtained from different quercetin concentrations [90]. The total flavonoid content of EQL was determined to be 39.05±0.005µg QE/mL. According to this result, it can be said that flavonoids constitute a large part of the phenolic content of EQL. It can be reported as an important result that it has the total flavonoid content close to the ginger plant (25.1 QE/mL), which is an important medicinal plant and has been studied many times [82]. In a similar study, the total flavonoid content of thyme plant was reported as 36.6 QE/mL [83].

3.2. Phenolic Composition of EQL

Identification of secondary metabolites is a fundamental step in understanding the biological activities of plant products [91]. These compounds play important roles in the protection and survival of plants against environmental stress factors. More importantly, secondary metabolites contain bioactive components that may provide pharmacological and therapeutic benefits for humans [92]. These metabolites isolated from the leaves of a plant can play a fundamental role in the development

of new drug candidates. A large portion of drugs based on natural products are derived from secondary metabolites. Therefore, the identification of these compounds contributes to the understanding of the clinical significance and therapeutic potential of plant sources [93].

Table 2. The determination of phytochemical profiles of quince leaf (*Cydonia Oblonga*)

No	Standard Compounds	ESI MOD	MRM	Ret. Time	R^2	Regression	Conc. (µg/L)
1	Quercetin	neg	301.10>151.00	6.091	0.999	y=13.7831x-146.951	85.5
2	Catechin	neg	291.10>139.00	4.958	0.999	y = 79.2933x -2406.22	2024.9
3	Vanillic acid	pos	168.80>93.00	6.026	0.998	y = 48.0522x -876.904	1121.8
4	Fumaric acid	neg	115.20>71.00	3.674	0.999	y = 20.2986x -762.592	486.7
5	Gallic acid	neg	169.20>125.00	4.134	0.999	y = 65.3835x -2699.84	15.3
6	Caffeic acid	neg	179.20>135.00	5.283	0.996	y = 124.785x -487.132	84.9
7	Hydroxycinnamic acid	neg	163.20>119.00	5.738	0.995	y = 13,1516x + 717.421	<LOD
8	Ellagic acid	neg	300.90>145.10	5.895	1.000	y = 5.25903x -1167.31	<LOD
9	Myricetin	neg	317.10>150.90	5.858	0.999	y =37.0934x + 2684.23	<LOD
10	Naringenin	neg	271.10>150.90	6.104	0,996	y = 317.241x + 33733.3	<LOD
11	Luteolin	neg	285.20>132.90	6.190	0,998	y = 34,6668x + 3721.79	<LOD
12	Kaempferol	neg	285.10>116,90	6.288	0,999	y = 2.63905x -206.494	<LOD
13	Hydroxybenzoic acid	neg	137.20>93.00	6.130	0.999	y = 735.804x -498.102	74.8

*Measurement uncertainty data of quantified compounds were given in the text.

The LC-MS/MS method is considered a powerful tool to identify and characterize biologically active compounds of plants. This method is used to identify various chemical compounds present in plants with high sensitivity and accuracy. Plant extracts are often complex mixtures, and LC-MS/MS helps distinguish and characterize specific compounds within these mixtures [93]. In the present study, LC- MS/MS was used to determine different phenolic compounds and the method was validated based on the formerly reported approach in the literature. To avoid plagiarism, the details of methodology were not discussed herein. To get more information please see the published papers in this journal [93-95]. Briefly, the method validation parameters were selected as specificity, linearity, repeatability, Limit of Detection (LOD) and Limit of Quantitation (LOQ). Measurement Uncertainty Assessment of the applied method was carried out based on former literature as well [96-98]. The main source of uncertainty comes from the calibration curve, and repeatability of the experiment. Detailed information of the methodology was given in former literature data. In the analyses performed, 13 different compounds were analyzed and the amounts of 7 of these compounds were quantitatively determined. The three phenolic structures with the highest amounts were seen to be catechin (2024.9 ± 80.9 µg/L), vanillic acid (1121.8 ± 39.3 µg/L) and fumaric acid (486.7 ± 16.5 µg/L). The total amount of these three compounds is 4 times more than the other phenolic compounds which are found as quercetin (85.5 ± 3.3 µg/L), gallic acid (15.3 ± 0.8 µg/L), caffeic acid (84.9 ± 3.9 µg/L) and hydroxybenzoic acid (74.7 ± 2.9 µg/L). Therefore, it is highly probable that the high biological activity shown by EQL is due to these identified compounds together with non-identified as herein.

3.3. Antioxidant Activity of EQL

3.3.1. Reducing Capability

Metal reduction assays measure the ability of antioxidants to transfer electrons. Antioxidants reduce oxidative stress by donating electrons to neutralize free radicals. This mechanism plays a critical role in preventing oxidative damage at the cellular level. The high metal reduction capacity of antioxidants may provide protective effects against metal-induced oxidative stress in biological systems [99]. For example, ferric ions (Fe^{3+}) and cupric ions (Cu^{2+}) can increase free radicals via the Fenton reaction. Antioxidants contribute to the reduction of oxidative stress by inhibiting the redox cycle of these metals [82]. Two different metal reduction methods (Fe^{3+} and Cu^{2+} reducing abilities) were used to determine the antioxidant capacity of quince leaf extract. The results obtained were compared with Trolox, a standard antioxidant. Measurements were made at three different concentrations (10-30 mg/mL) for both the plant extract and Trolox, which was found to have higher metal reduction capacity at all concentrations studied (Table 3 and Figure 1).

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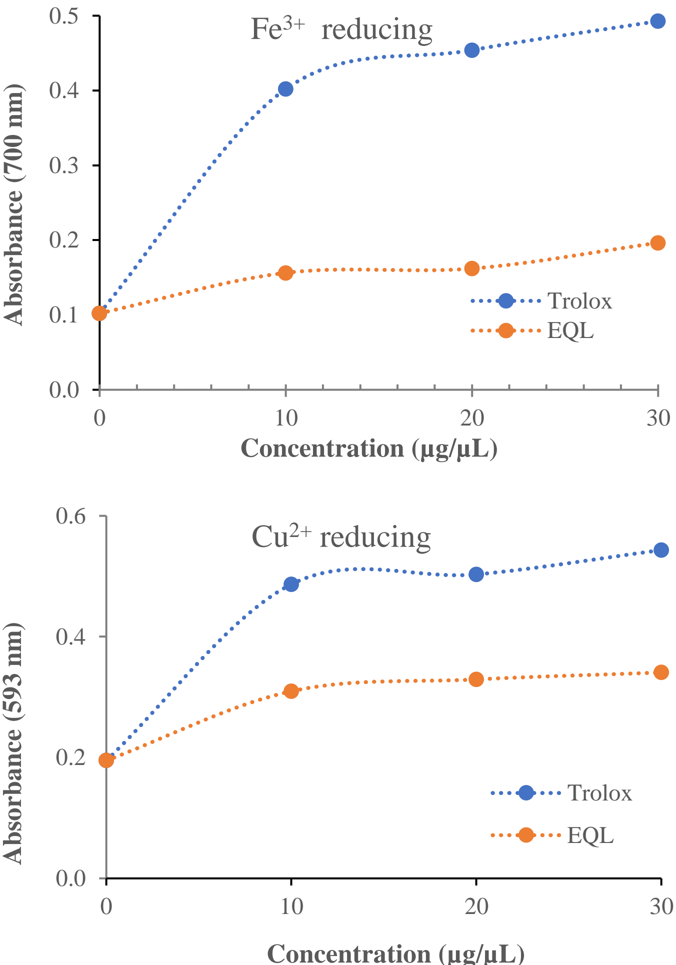

Figure 1. Ferric ions (Fe^{3+}) and cupric ions (Cu^{2+}) reducing abilities of ethanol extract of quince (*Cydonia oblonga*) leaves (EQL) and Trolox.

These results indicate that the total redox potential of antioxidant compounds in quince leaf extract may be limited. However, considering the diversity of phenolic and flavonoid compounds found in the plant, this does not exclude the contribution to other biological activities of the extract.

3.3.2. Radical Scavenging Capability

Radical scavenging activity is one of the main parameters used to measure the biological activity of a plant. It directly indicates the capacity of the plant extract to capture free radicals and protect against oxidative stress [100]. Plants with high radical scavenging activities are important candidates for the development of health-supporting products. Phenolic compounds and flavonoids in plant extracts are biomolecules with strong antioxidant properties. Radical scavenging activities are generally directly related to the amount of these compounds. Therefore, the investigation of radical scavenging activities allows us to better understand the biological importance of phenolic and flavonoid content. Herbal products offer sustainable and safe sources that can be used as natural antioxidants. The study of radical scavenging activities enables the discovery of new and effective natural antioxidant sources [89]. Two different radicals, DPPH and ABTS, were used to determine the radical scavenging activities of quince leaves and the standard antioxidant compound Trolox. 0.2 mg/mL solutions of the sample and standard compound were studied and the percentage of radical

scavenging of both structures was determined. It was observed that quince leaves had the ability to scavenge both radical structures, but Trolox was far behind. The results are given in Table 4

.

Table 3. Fe^{3+} and Cu^{2+} reducing abilities at 30 µg/mL and IC_{50} (mg/mL) values of DPPH$^{•}$ and ABTS$^{•+}$ scavenging effects of ethanol extract of quince (*Cydonia oblonga*) leaves (EQL) and Trolox

Antioxidants	Fe^{3+} reducing		Cu^{2+} reducing		DPPH scavenging	ABTS scavenging
	λ_{700}	r^2	λ_{593}	r^2	IC_{50}	IC_{50}
Trolox	0.4927	0.9929	0.5433	0.9435	0.123	0.125
QEL	0.1964	0.8542	0.2407	0.9772	0.776	0.762

3.4. Cholinesterase Enzymes Inhibition Abilities of EQL

AChE and BChE enzymes play important roles in synaptic transmission and in the regulation of cholinergic pathways in the central nervous system. Especially in neurodegenerative diseases such as Alzheimer's disease, excessive activity of AChE and BChE reduces acetylcholine levels, leading to cognitive disorders [98]. In this context, the alcohol extract of the studied plant may contribute to the correction of imbalances in the cholinergic system thanks to its high inhibitory effect. These results demonstrate the potential of quince leaf extract as a natural AChE and BChE inhibitor (Figure 2). Considering that the use of synthetic inhibitors such as Tacrine has some side effects, the safety profile and efficacy of this herbal alternative may offer a significant advantage. In the future, further biological activity testing and clinical studies of this herbal inhibitor will further clarify its potential for use in the treatment of Alzheimer's and other cholinergic system disorders.

In this study, the inhibitory effects of quince leaf alcohol extract on AChE and BChE enzymes were evaluated. The results showed that the plant extract had a high effect in inhibiting both enzymes and this effect was stronger than the Tacrine as standard inhibitor. Especially, in terms of IC_{50} values, quince leaf extract exhibited lower IC_{50} values for both AChE and BChE. These findings strongly suggest the potential of the plant extract to inhibit these enzymes. Natural inhibitors with lower IC_{50} values may provide more targeted and effective treatment by suppressing the same enzyme activity at lower concentrations. In studies conducted within the scope of acetylcholinesterase inhibition, quince leaf extract has a lower IC_{50} value (1.303 µg/mL, r^2: 0.9436) than Tacrine (3.194 µg/mL, r^2: 0.9952). Similar inhibition results were also observed for the butyrylcholinesterase enzyme. Quince leaf extract has a lower IC_{50} value (1.053 µg/mL, r^2: 0.9102) than Tacrine (2.510 µg/mL, r^2: 0.9668). These findings clearly showed that quince leaf extract has potential to offer a more effective and safer natural inhibition and it can be use as alternative in the treatment of neurodegenerative diseases after further required researches. These findings emphasize that the use of natural products in pharmaceutical research should be increased and inhibitors obtained from natural sources should be seriously evaluated for clinical applications.

α-Glycosidase is a key enzyme that plays a role in the breakdown of carbohydrates into glucose and other simple carbohydrate. Inhibitors of this enzyme are used to control postprandial blood glucose levels by delaying glucose absorption [101]. Drugs such as Acarbose, which are frequently used in diabetes treatment, are inhibitors of this enzyme. However, due to the gastrointestinal side effects of Acarbose (bloating, gas, diarrhea), compliance with treatment may be low [102,103]. As can see in Figure 2 and Table 4, the inhibitory effect of alcohol extract of quince leaves on α-glycosidase enzyme was investigated and the results showed that this plant extract had a lower IC_{50} value (0.6 µg/mL, r^2: 0.9638) compared to the standard inhibitor Acarbose (3.238 µg/mL). The lower IC_{50} value suggests that this plant is effective at less concentration to inhibit α-glycosidase and therefore has a stronger inhibitory potential than Acarbose. The fact that quince leaf extract has a lower IC_{50} value than Acarbose indicates that this herbal inhibitor may be more effective at lower doses. This is important for the development of new natural alternatives that can provide better glycemic control with fewer side effects in diabetes treatment. The alcohol extract of quince leaves was determined as a strong

inhibitor of the α-glycosidase enzyme. Having a lower IC_{50} value than Acarbose shows that this plant can be used as a natural and effective alternative inhibitor in diabetes treatment. However, further in vivo and clinical studies are required for clinical verification of this effect. These findings emphasize the potential of herbal products to offer innovative solutions in diabetes treatment. To determine these solutions, it is important to isolate and structure determine the secondary metabolites found in the plant structure and obtain them preoperatively. As a result of investigating the inhibitory effects of these structures obtained preoperatively on the α-glycosidase enzyme, the compound or compounds that inhibit the enzyme at a high level can be determined.

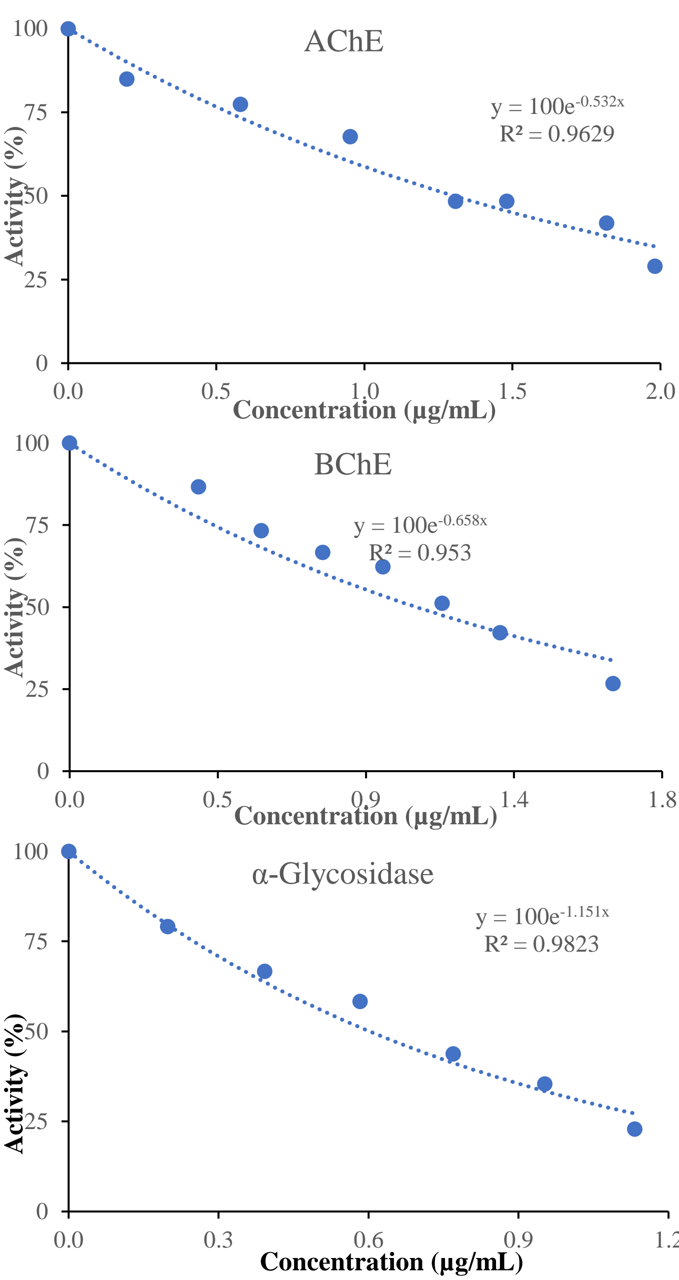

Figure 2. Half maximal inhibition concentration (IC_{50}) graphs of ethanol extract of quince (*Cydonia oblonga*) leaves (EQL)

Table 4. Enzyme inhibition results ethanol extract of quince (*Cydonia oblonga*) leaves (EQL)

Enzyme	EQL		Standard inhibitors	
	IC50 (µg/mL)	**r²**	**IC50** (µg/mL)	**r²**
Acetylcholinesterase	1.3	0.9436	3.2	0.9952
Butyrylcholinesterase	1.1	0.9102	2.5	0.9668
α-Glycosidase	0.6	0.9638	3.2	0.9856

These findings emphasize the potential of herbal products to offer innovative solutions in the treatment of diabetes. To determine these solutions, it is important to isolate and structure determine the secondary metabolites found in the plant structure and obtain them preoperatively. As a result of investigating the inhibitory effects of these structures obtained preoperatively on the α-glycosidase enzyme, the compound or compounds that inhibit the enzyme to a high degree can be determined. This result shows that quince leaf extract is five times more effective than Acarbose, which is a standard inhibitor and also used as a drug, on the α-glycosidase enzyme. In addition, the IC_{50} value of quince leaf extract being below 1 µg/mL is another remarkable result. It is an extremely important result that a plant extract inhibits the α-glycosidase enzyme with an IC_{50} value at the nanogram level. This degree of inhibition as a pure compound has been reported [104] in the literature, but it is the first time that a plant extract has an IC_{50} value at the ng/mL level.

3.6. Molecular Docking Studies

In this study, the interactions of the phenolic structure catechin hydrate, which constitutes the phytochemically highest amount of quince leaf (*Cydonia oblonga*) extract determined by LC-MS-MS method, with AChE and α-glycosidase enzymes were determined using molecular docking method. The studies were carried out using the Schrödinger Molecular Modeling Suite Maestro 13.5 program (https://doi.org/10.1002/slct.202303054). In the first stage of molecular docking studies, molecular structures of catechin hydrate and Tacrine, Acarbose, which are clinical inhibitors of AChE and AG enzymes, respectively, were created using SMILES codes obtained from Pubchem internet database and optimization studies were performed using Maestro's LigPrep software (Tables 5 and 6) [105-108].

Table 5. Scores of docking and binding energies belonging to the compounds for enzymes, AChE, and α-glycosidase

Ligand	**PubChem CID code**	**References**
Catechin	CID107957	https://pubchem.ncbi.nlm.nih.gov/compound/107957
Tacrine	CID1935	https://pubchem.ncbi.nlm.nih.gov/compound/1935
Acarbose	CID41774	https://pubchem.ncbi.nlm.nih.gov/compound/41774

Enzyme structures were taken from the RCSB Protein Database website (PDB identifiers; AChE: 4TVK, α-glycosidase: 3L4Y). Protein optimization studies were performed using the protein preparation wizard tool in Schrodinger software version 13.5.128. The studies continued with the preparation of receptor grids for ligand docking analysis. The preparation of receptor grids allows the determination of the position and size of the active site of the protein. These studies were performed using the receptor grid generation tool in Maestro 13.5, Schrödinger Suite 2022-3. The potential interactions and binding affinities of the molecules with each enzyme were determined using ligand docking software of the Schrödinger Molecular Modeling Suite (Maestro Version 13.5) [106-108].

The docking and binding energy scores of the catechin hydrate obtained as a result of the study and the reference AChE enzyme inhibitor Tacrine and α-glycosidase enzyme inhibitor Acarbose are given in Table 5. In addition, the pocket grid size of the protein, which is the summary result of the

study, and the residues and distances (Å) involved in hydrophobic interactions are given in Table 6 and the 2D and 3D-zoomed docking images are given in Figure 3.

Table 6. Summative results of docking studies of the molecules identified for extract with enzymes

No	Ligand	Pocket grid dimension of protein	Residues involved in hydrophobic interactions and Distances (Å)
1	Catechin-AChE	Grid Box Center coordinates center_**x** = -52.437 center_**y** = 30.244 center_**z** = -43.435 Grid Box Dimension (Å) size_**x** = 20 size_**y** = 20 size_**z** = 20	TYR334 (5.45) pi stacking, PHE330 (4.48) pi stacking, ASP72 (1.82) H Bond
2	Tacrine-AChE	Grid Box Center coordinates center_**x** = -52.437 center_**y** = 30.244 center_**z** = -43.435 Grid Box Dimension (Å) size_**x** = 20 size_**y** = 20 size_**z** = 20	PHE330 (3.62, 3.96) pi stacking, TRP84 (4.00, 3.62) pi stacking, HIS440 (1.92) H bond, PHE330 (4.05) pi cation,
3	Catechin -α-Glycosidase	Grid Box Center coordinates center_**x** = -1.961 center_**y** = -19.186 center_**z** = -21.048 Gr,d box dimension (Å) Size **x** = 20 Size **y** = 20 Size **z** = 20	ASP327 (2.11) H Bond
4	Acarbose- α-Glycosidase	Grid Box Center coordinates center_**x** = -1.961 center_**y** = -19.186 center_**z** = -21.048 Grid Box Dimension (Å) size_**x** = 20 size_**y** = 20 size_**z** = 20	ASP542 (1.70, 1.85) H bond, ARG526 (1.74, 1.95) H bond, ASP203 (1.75, 1.83) H Bond, ASP327 (1.85) H bond

Enzyme structures were taken from the RCSB Protein Database website (PDB identifiers; AChE: 4TVK, α-glucosidase: 3L4Y). Protein optimization studies were performed using the protein preparation wizard tool in Schrodinger software version 13.5.128. The studies continued with the preparation of receptor grids for ligand docking analysis. The preparation of receptor grids allows the determination of the position and size of the active site of the protein. These studies were performed using the receptor grid generation tool in Maestro 13.5, Schrödinger Suite 2022-3.

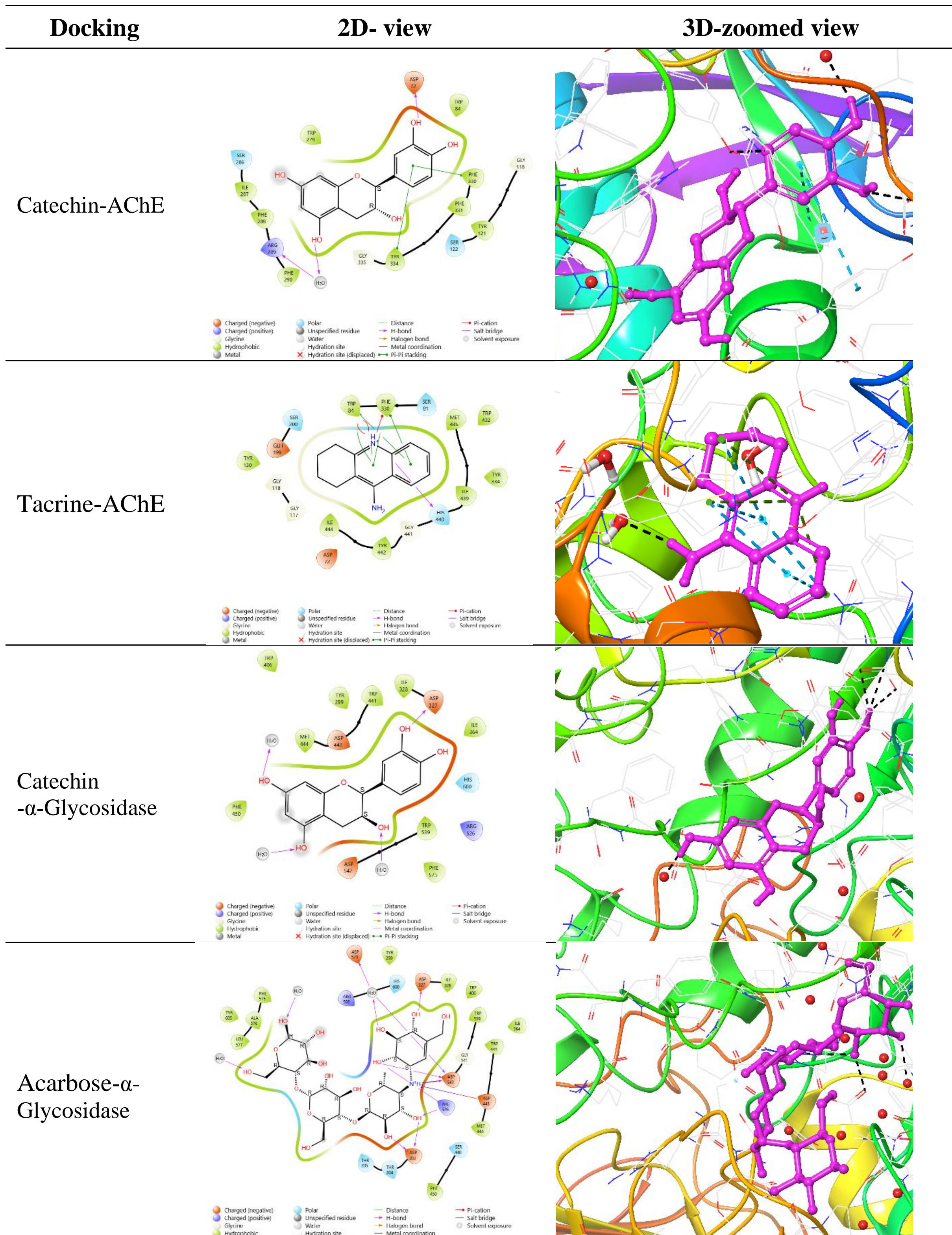

Figure 3. Molecular docking 2D and 3D ligand protein interaction of catechin hydrate-AChE and catechin hydrate-α-glycosidase complexes.

The potential interactions and binding affinities of the molecules with each enzyme were determined using ligand docking software of the Schrödinger Molecular Modeling Suite (Maestro Version 13.5). The docking and binding energy scores of the catechin hydrate obtained as a result of the study and the reference AChE enzyme inhibitor Tacrine and α-glycosidase enzyme inhibitor Acarbose are given in Table 2. In addition, the pocket grid size of the protein, which is the summary result of the study, and the residues and distances (Å) involved in hydrophobic interactions are given in Table 3 and the 2D and 3D-zoomed docking images are given in Table 7.

4. Conclusions

Ethanol extract of quince tree leaves was prepared. Total phenolic and flavonoid amounts of this extract were determined and it was observed that the phenolic content of quince leaves was largely formed by flavonoids. Polyphenol content of the extract was determined by LC-MS/MS method. The antioxidant activity of the extract was investigated by metal reduction and radical scavenging methods. The results were compared with Trolox, a standard antioxidant. It was understood that the antioxidant activity of ethanol extract of plant leaves was less than Trolox. In α-glycosidase inhibition studies, it was determined that the extract inhibited this enzyme with an IC_{50} value at nanogram level. We report this as an important result in terms of antidiabetic studies. It was also seen that the extract inhibited AChE and BChE enzymes at a higher rate than the standard inhibitor.

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