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Phytochemical Properties, Antioxidant and in Vitro/in Silico Anti-Acetylcholinesterase Activities of *Hypericum heterophyllum* Leaf from Türkiye

Cennet Yaman[1]*
https://orcid.org/0000-0002-2364-8171

Ramazan Erenler[2]
https://orcid.org/0000-0002-0505-3190

Mehmet Nuri Atalar[2, 3]
https://orcid.org/ 0000-0003-2993-2605

Şevki Adem[4]
https://orcid.org/ 0000-0003-2146-5870

Ufuk Koca Çalişkan[5]
https://orcid.org/ 0000-0002-5216-7588

[1]Yozgat Bozok University, Faculty of Agriculture, Department of Field Crops, Yozgat, Türkiye; [2]Iğdır University, Faculty of Health Sciences, Iğdır, Türkiye; [3]Iğdır University, Research Laboratory Application and Research Center, Iğdır, Türkiye; [4]Çankiri Karatekin University, Department of Chemistry, Cankiri, Türkiye; [5]Gazi University, Faculty of Pharmacy, Pharmacognosy, Ankara, Türkiye.

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*Correspondence: cennet.yaman@yobu.edu.tr; Tel.: +09 0354 242 10 28 (C.Y.).

HIGHLIGHTS

- *Hypericum* extracts have been used in the traditional medicine.
- *H. heterophyllum* extracts contained various bioactive compounds.
- The effect of the different solvent extraction was revealed.
- The solvent type displayed major effect on the various biological activities.

Abstract: Methanol, ethanol, acetone and chloroform extracts of powdered *Hypericum heterophyllum* prepared from leafs were investigated for their (i) phytochemical constituents, which was evaluated by LC-ESI-MS/MS and various known total bioactive methods; (ii) antioxidant capacity, which was evaluated by several in vitro assays such as DPPH, ABTS, H_2O_2, superoxide radical, and metal chelating; (iii) acetylcholinesterase (AChE) inhibitory activity; (iv) active constituents of the extracts with AChE in silico docking. Phytochemical analysis revealed that none of the extracts contained hypericin, pseudohypericin and hyperforin. Among the identified compounds, ethanol and acetone extracts had the highest number of compounds. The highest value of total phenolic content (132.7 mg GAE/g) was obtained with methanol extract, whereas the highest values of total flavonoid (61.8 mg QE/g) and flavanol (0.89 mg CE/g) content were recorded in acetone extract. Methanol extract displayed the most DPPH activity (24.9 mg TE/g), but ethanol extract (59.8 mg TE/g) showed the highest ABTS activity. Acetone extract displayed the best hydroxyl and superoxide radical scavenging activities with the values of 351.5 mg AAE/g, and 197.3 mg TE/g respectively. The chloroform extract exhibited the best AChE inhibition activity (11.07 µg/ml). In the docking study, quercetin-7-O-glucoside, diosgenin, and quercetin-3-glucoside were calculated to have a stronger

MolDock Score than the reference ligand Donepezil. Our docking results have indicated that extracts contain compounds with a high affinity against the acetylcholinesterase enzyme. These data suggest that these extracts can be potentially important antioxidant supplements and drug candidates for AChE inhibition activity.

Keywords: *Hypericum heterophyllum*; phytochemical; biological activity; in silico docking; solvent.

INTRODUCTION

Hypericum genus belonging to the Hypericaceae (previously *Clusiaceae* or Guttiferae) family consists of approximately 469 species distributed throughout the world [1]. This genus is represented by total of 96 species in the flora of Türkiye, and 46 of which are endemic [2].

Hypericum species dramatically continues to increase their economic importance due to their wide application in different fields and mainly in clinical studies. Mainly, *H. perforatum* is known as a natural source for therapeutic purposes of several diseases such as mild to moderate depression, wound healing among other health concerns, such as antioxidant and anticholinesterase activities [3]. *H. perforatum* L. known as St. John's wort in English, been used in traditional medicine for thousands of years, specifically the focus of interest clearly being on its potential as an herbal antidepressant. The annual sales value of different forms of herbal antidepressant products exceeded $6 million in the USA markets in 2016; *Hypericum* preparations are reported to significantly reduce the cost of depression therapy as a substitute for standard drugs for mild depressions [4]. Therefore, it is among the best-selling supplements in the United States and several European countries in treating mild depression [5, 6]. Genus *Hypericum* has a wide variety of secondary metabolites such as napthodianthrones (hypericin, pseudohypericin, protohypericin etc.), flavonoids (campherol, quercetin, rutin, luteolin, hyperin, hyperoside), phenolic acids, phloroglucinols (hyperforin, adhyperphorin etc.), xanthones and essential oils [1, 7]. There are numbers of studies reporting phytochemicals of some *Hypericum* species and their antioxidant potentials [3, 6].

Several biochemical reactions in living organisms generate reactive oxygen species, including hydroxyl (OH), peroxyl and superoxide (O_2^-) radicals, and these generally results in disrupting the structure of crucial biomolecules such as degradation of protein and carbohydrates, oxidation of DNA, and lipid peroxidation. If they are not effectively scavenged or blocked by the organism, they cause various disease as well as aging potentials [8]. The harm of excessive free radicals in human body has been investigated by many researchers, and their roles in diseases are well established [9].Comparable to free radicals, due to their ability to encourage electron transfer, ferrous ions also promote the deterioration of these molecules, which catalyzes both protein and lipid oxidation [10]. Virtuous chelation of ferrous ions might strongly provide excellent antioxidative activity by retarding metal-catalyzed oxidation [11]. The injurious effects of free radicals and ferrous ions can be reduced or eliminated by antioxidants mostly natural radical scavenging products [12, 13]. One of the largest natural sources of antioxidants is obviously phytochemicals or whole extracts of plants [14]. Various parameters can have a massive impact on recovering of antioxidant compounds from a particular plant, such as extraction and solvent diversity specifically. Consequently, various antioxidant compounds might or might not be extractable in a particular solvent due to the chemical characteristics and polarities [15]. Polar solvents such as ethanol, methanol, and acetone are frequently used for recovering polyphenols from plants. Acetone is a favorable solvent for higher molecular weight flavanols, whereas methanol is more effective for extraction of lower molecular weight polyphenols, and ethanol has been usually found as a good solvent for polyphenol extraction [16]. In addition, phytochemical contents and antioxidant activities of plants differ according to their plant parts such as root, flower, stem, leaf, seed, fruit, and others [17,18].

Plants and secondary metabolites in plants have been used for therapeutic purposes in the treatment of many diseases, especially Alzheimer's disease, and various biochemical effects have been shown as therapeutic agents [19]. One of the important factors in the emergence of Alzheimer's disease is the cholinergic hypothesis. In the cholinergic hypothesis, inhibition of cholinesterase (acetylcholinesterase and butyrylcholinesterase) enzymes to prevent the reduction of acetylcholine in the brain is important in the treatment of Alzheimer's disease [20, 21]. Thus, it is important to design and identify new plant metabolites with inhibitory effects on AChE.

Hypericum species have considerable use in traditional and modern medicine, as well as economic importance in many industries such as food, cosmetics, pharmacy, aromatherapy. However, there is not enough research on many species yet. Therefore, the aim of this study is to determine and compared the effect of various solvents on total bioactive compounds and free radicals of naturally grown *Hypericum heterophyllum* leaf, an endemic for Türkiye. Furthermore, another significant goal of this study was to

contribute to the selection of suitable extraction solvent to obtain major compounds for the potential commercial applications. To the best of our knowledge, this is the first comprehensive report on effect of various solvents on phytochemical compounds, total bioactive contents, free radical scavenging, metal chelating activities and acetylcholinesterase inhibition of *H. heterophyllum* leaf*.* Further, in silico docking analysis of active compounds identified in liquid chromatography electrospray ionization tandem mass spectrometric (LC-ESI-MS/MS) with AChE was performed.

MATERIAL AND METHODS

Plant Material

Flowering aerial parts of *H. heterophyllum* Vent., endemic speices for Türkiye was collected between 11:00 am. and 13:00 pm. from their natural habitats (39º46'42, 34º47'51, 1332, Yozgat-Türkiye). The leaf parts of the plant were separated, and dried under shade at 20 ± 2 ºC for analysis. The plant material were identified by Prof. Dr. Osman Tugay (Department of Pharmaceutical Botany, Faculty of Pharmacy, Selçuk University) and the voucher specimen has been deposited at KNYA Herbarium of the Selçuk University, Faculty of Science, Konya, Türkiye (Voucher No: 28283).

Chemicals

Folin–Ciocalteu reagent, quercetin, gallic acid, 2,2 diphenyl-1-picrylhydrazyl (DPPH), trolox, butylated hydroxytoluene (BHT), potassium acetate, aluminium nitrate, hydrochloric acid (HCl), hydrogen peroxide solution (H_2O_2), phenazine methosulphate (PMS), β-nicotinamide adenine dinucleotide (NADH), nitroblue tetrazolium (NBT), ethylenediaminetetraacetic acid (EDTA) and 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS) were from Sigma-Aldrich. Sodium carbonate anhydrous, $FeCl_2$, methanol, chloroform, acetone and ethanol were from Merck, and 4-(Dimethylamino) cinnamaldehyde (DMACA) were from Alfa Aesar.

Preparation of *Hypericum* Extracts and Phytochemical Compounds by LC-ESI-MS/MS

The leafs of *H. heterophyllum* were used for the extraction, and were dried under shade and mechanically ground with a blender. The samples (4 g) of grounded plant materials were individually macerated with 40 mL of solvent with methanol, ethanol, acetone and chloroform at 40°C for 24 h. Methanol, ethanol, acetone and chloroform were then removed with a rotary evaporator at temperature bellow 40°C to obtain extracts with yields of 19.1%, 19.6%, 8.6% and 9.0%(w/w), respectively. Extracts obtained using organic solvents were dissolved in acetone and then filtered. Methanol extract was dissolved in grade water.

The concentration of various solvent extracts of *Hypericum heterophyllum* leaf was adjusted as 2 mg/mL. Subsequently, the solution was filtered through 0.45 µm filters and transferred into vials prior to LC-MS/MS analysis. The phytochemical compound analysis of the prepared plant solutions was carried out using the LC-MS/MS device with the multiple reaction monitoring (MRM) method developed by Yırtici and coauthors 4.0 µL of the sample was injected, Poroshell 120 EC-C18 (100 mm × 4.6 mm I.D., 2.7 µm) was used for simultaneous separation of phytochemical compounds and 0.1% formic acid and 5mM ammonium formate in water and methanol were used as mobile phases [22].

Total Bioactive Contents

Total phenolic contents

The total phenolic content (TPC) was measured using the Folin-Ciocalteu method [23]. Briefly, 0.2 mL Folin-Ciocalteu and 9 mL distilled water were added into the 200 µL of each sample extracts. Finally, 0.6 mL of 20% sodium carbonate solution was added, and the total volume was adjusted to 10 mL. After the mixtures were waited in the dark for 2 hours at room temperature, absorbance measurement was read at 760 nm. The results were represented as mg gallic acid equivalent (GAE)/ g extract. The calibration curve was created using nine different concentrations of the gallic acid standard ($y = 0.0087x + 0.0166$; $R^2 = 0.9973$).

Total flavonoid contents

The total flavonoid content (TFC) was measured using the method developed by Arvouet-Grand and coauthors [24], with minor modifications. 10% aluminum nitrate (100 µL) and 1 M potassium acetate (100 µL) were added into the 200 µL of each sample extracts. The total volume was adjusted to 5 mL with 99% ethanol.

After the mixtures were incubated in the dark for 40 min at room temperature, absorbance measurement was performed at 417 nm. The total flavonoid contents of the extracts were calculated as mg quercetin equivalent (QE)/ g extract using calibration curve ($y = 0.0057x + 0.0151$; $R^2 = 0.9965$) of quercetin standard.

Total flavanol content

The total flavanol content (TFLC) was analyzed using the method developed by Quettier-Deleu and coauthors [25] with slight modification. The sample (250 µL) was added to 5 mL of 0.1% DMACA (*p*-dimethylaminocinnamaldehyde) in methanol:HCl (3:1) reagent. After the mixtures were incubated for 10 min at room temperature, absorbance measurement was performed at 417 nm. According to the standard graph of catechin ($y = 0.0105x - 0.0037$; $R^2 = 0.9982$), total flavanol contents of the extracts were calculated as µg catechin equivalent (CE)/ g extract.

Radical Scavenging Activity

DPPH free radical scavenging activity

The effect of various solvent extracts of the *H. heterophyllum* leaf on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was tested according to Ceylan and Alıc [26]. The sample (0.2 mL) was added to a 3.2 mL of a 0.004% methanol solution of DPPH. After the mixtures were incubated in the dark for 30 min at room temperature, absorbance measurement was performed at 517 nm. Butylated hydroxytoluene (BHT) as standard was used. According to the graph of trolox standard ($y = 0.0057x + 0.0089$; $R^2 = 0.9998$), the DPPH radical scavenging activities of the extracts were expressed as mg trolox equivalent (TE)/ g extract.

ABTS free radical scavenging activity

ABTS (2,2-azino-bis (3-ethylbenzothiazloine-6-sulfonic acid) was used for evaluation of radical cation scavenging activity according to the method described by Yaman [27]. In this method, the stock solution of $ABTS^{\bullet+}$ was obtained directly by reaction of 30 mg ABTS and 6.6 mg potassium per sulphate in 7.8 mL of distilled water, and allowing the mixture to stand for 12–16 h in dark at the room temperature. Then, the ABTS solution was diluted with methanol to an absorbance of 0.700 ± 0.020 at 734 nm using a UV visible spectrophotometer. ABTS solution (2.8 mL) was added on 100 µL to sample solutions, and mixed. The absorbances were recorded at 734 nm after 30 min incubation at room temperature in the dark. BHT as standard was used. The ABTS free radical scavenging activities of all tested extracts were calculated as mg trolox equivalent (TE)/ g extract using calibration curve ($y = 0.0023x + 0.0243$; $R^2 = 0.9983$) of quercetin standard.

Hydroxyl radical (*OH*)-scavenging activity

The hydrogen peroxide scavenging activity was measured using the phosphate buffer (0.04 M, pH = 7.4) by Ruch and coauthors [28] with minor modification. Briefly, 2.4 mL of buffer solution (0.1 M, pH = 7.4 phosphate buffer) and 1 mL of H_2O_2 (40 mM) prepared phosphate buffer were added into 100 µL of sample solution. Absorbance measures were read at 230 nm after 10 min incubation. The H_2O_2 scavenging activity was expressed as equivalents of ascorbic acid (AAE)/ g extract according to the equation obtained from the standard ascorbic acid graph ($y = 0.0028x + 0.0135$; $R^2 = 0.999$).

Superoxide radical (O_2^-)-scavenging activity

Superoxide radical scavenging activity was determinated according to the method described by de Gaulejac and coauthors [29] with minor modification. Superoxide radicals were generated by oxidation of NADH and assayed by the reduction of NBT. Briefly, 1 mL of sample solution (2 mg /mL) were mixed with 1 mL of NBT (156 µM in 0.1 M, pH=7.4 phosphate buffer) and 1 mL NADH (468 µM in 0.1 M, pH=7.4 phosphate buffer) solution. The reaction was started by the addition of 1 mL of PMS (60 µM in 0.1 M, pH=7.4 phosphate buffer) to the mixture, which was incubated at 25°C for 5 min., and then the absorbance was measured at 560 nm. The superoxide radical scavenging activities of all tested extracts were expressed as equivalents of trolox (TE)/ g extract according to the equation obtained from the standard trolox graph ($y = 0.0032x – 0.0545$; $R^2 = 0.9956$).

Metal Chelating Activity on Ferrous Ions

The metal chelating activities on ferrous ions of tested extracts were measured by the method described by Dinis and coauthors [30]. The sample solution (1 mL, 2 mg/mL) was added to $FeCl_2$ solution (50 µL, 2 mM), and mixed with 3.7 mL distilled water. After incubating for 30 min, the reaction was initiated by the addition of 0.2 mL ferrozine (5 mM). The absorbance of all tested extracts was read at 562 nm after 10 min incubation at room temperature. The metal chelating activities of the extracts was expressed as equivalents of EDTA according to the equation obtained from the standard EDTA graph ($y = 0.0145x + 0.13$; $R^2 = 0.9955$).

In vitro and in silico AChE Inhibition Studies

AChE activity was determined spectroscopically at 412 nm as a result of the reaction using acetylthiocholine iodide as substrate [31] and percent inhibition and IC_{50} values were calculated to determine inhibition for each extract [32].

The docking calculations were performed using MolegroVirtual Docker software [33]. The crystal structure of the acetylcholinesterase coded 4EY7 at RCSB PDB was retrieved [34]. While the protein was imported into the program, water molecules and ions not required for activity were removed. Missing residues at the crystal structure were repaired and optimized with neighbors' amino acids. The 3D Conformers of compounds were downloaded in SDF format from the PubChem database. They were imported to the docking software. Donepezil in the crystal structure was re-docked, and parameters giving an RMSD value below two were selected for later docking protocol. We settled Donepezil at the center of the binding site, selected a 15 Å radius for the cavity, carry out ten runs for each compound. Energy minimization and optimization of hydrogen bonds were performed after docking. The best docking poses for each phytochemical were chosen, and their 2D binding modes were analyzed using Chimera 1.15 and Discovery Studio 2021 Client [35].

Statistical Analysis

All data was statistically analysed using one-way ANOVA, and comparison of the means was carried out by Duncan's multiple range tests at a significance level of 0.05 and the data were given as the mean ± standard deviation (SD). Statistical analysis was performed using the SPSS 20.0 software package.

RESULTS AND DISCUSSION

Phytochemical Analysis

Recently, phenolic and polyphenolic compounds from natural resources have been a focus of interest by many industries and scientists owing to their use in the human diet, their notable antioxidant activities that protect human body's specific tissues against oxidative stress induced diseases, and medical uses [36, 37]. Many scientific reports have been published on the determination of botanical components by LC–ESI–MS/MS technique [38, 39].

The applied method allowed the identification and quantification of 50 phenolic compounds in methanol, ethanol, acetone and chloroform extracts of *H. heterophyllum* leafs. The amount of all bioactive compounds of *H. heterophyllum* leaf significantly varied in various solvent extracts. The changes in the chemical compounds are illustrated in Table 1. Although there are few studies on the bioactive compounds of *H. heterophyllum* [27, 38-41], this is the first comprehensive investigation, to our knowledge, on phytochemicals in the various organic solvent extracts of *H. heterophyllum* leaf.

The methanol extract of *H. heterophyllum* leaf included the shikimic acid (0.146 mg/g extract) as a major product. However, ethanol extract contained the chlorogenic acid (11.5 mg/g extract) as a chief compound. Moreover, shikimic acid (5.6 mg/g extract), hyperocide (1.4 mg/g extract), kaempferol-3-glucoside (0.26 mg/g extract), quercetin (0.20 mg/g extract) were detected as the most concentrated compounds. In concerning the acetone extract, the most concentrated compounds were detected in this extract. Chlorogenic acid (20.7 mg/g extract) was the major compound, shikimic acid (9.3 mg/g extract), hyperocide (2.2 mg/g extract) were also detected in high concentration. Chloroform extract included the only two compounds which were sinapic acid and flavon. Interestingly, these two compounds were more abundant in chloroform extract than in other extracts. Pandi and Kalappan [39] stated that sinapic acid has pharmacologically the potential use in many biological activities.

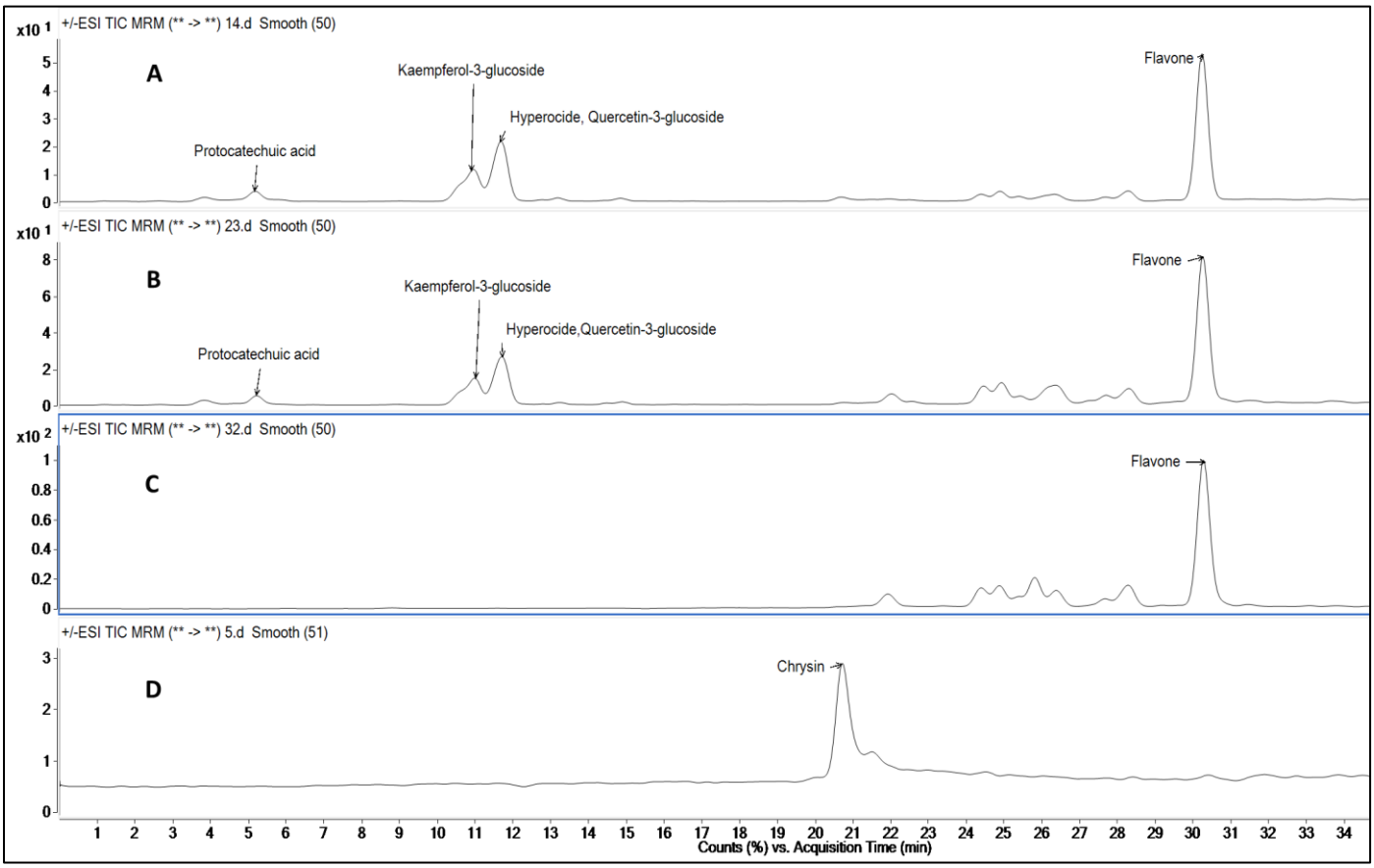

Figure 1. LC–ESI–MS/MS MRM chromatograms of the acetone, ethanol, and chloroform and methanol extracts of *Hypericum heterophyllum* leaf (A, B, C, and D chromatograms, top to bottom, respectively)

In all identified compounds, important *Hypericum* compounds such as hypericin, pseudohypericin, and hyperforin were specified in none extracts (Table 1). Similarly, Ayan and Çırak [41] reported that the hypericin and pseudohypericin could not be found in leaf parts of *H. heterophyllum*, even in its stem and flower parts. Hazman and coauthors [39] stated that both compounds were not found in methanol and ethanol extracts of *H. heterophyllum*. On the contrary, Smelcerovic and coauthors [38] expressed that hypericin, pseudohypericin, hyperforin and hyperoside were determined in ethanol extracts of all studied *Hypericum* species which grow in Türkiye (*H. heterophyllum, H. androsaemum, H. perforatum, H. triquetrifolium, H. aviculariifolium, H. bithynicum, H. montbretii, H. orientale, H. hyssopifolium, H. scabrum, H. hirsutum, H. linarioides, H. nummularioides, H. pruinatum, H. montanum, H. origanifolium*).

Table 1. Phytochemical compounds in various solvent extracts of *H. heterophyllum* leaf (mg/g)

Compounds	Methanol	Ethanol	Acetone	Cloroform
Ascorbic Acid	nd	nd	nd	nd
Shikimic acid	0.146	5.592	9.275	nd
Gallic acid	nd	0.036	0.028	nd
Protocatechuic acid	nd	0.054	0.085	nd
Gentisic acid	nd	nd	nd	nd
Catechin	nd	nd	nd	nd
4-Hydroxybenzoic acid	nd	nd	nd	nd
Chlorogenic acid	nd	11.465	20.698	nd
4-Hydroxybenzaldehyde	nd	nd	nd	nd
Vanillic acid	nd	nd	nd	nd

Cont. Table 1

Caffeic Acid	nd	0.014	0.014	nd
Epicatechin	nd	nd	nd	nd
Syringic acid	nd	nd	nd	nd
P-coumaric acid	nd	nd	nd	nd
Taxifolin	nd	nd	nd	nd
Polydatine	nd	nd	nd	nd
Trans-ferulic acid	nd	nd	nd	nd
Sinapic acid	nd	0.025	0.078	0.156
Quercimeritrin	nd	nd	nd	nd
Coumarin	nd	nd	nd	nd
Scutellarin	nd	0.204	0.177	nd
O-coumaric acid	nd	nd	nd	nd
Cynarin	nd	0.350	0.443	nd
Protocatechuic ethyl ester	nd	nd	nd	nd
Quercetin-3-glucoside	nd	0.842	0.912	nd
Hyperoside	nd	1.437	2.241	nd
Rutin	nd	nd	nd	nd
Isoquercitrin	nd	nd	nd	nd
Resveratrol	nd	nd	nd	nd
Naringin	nd	nd	nd	nd
Rosmarinic acid	nd	nd	nd	nd
Quercetin-3-D-xyloside	nd	nd	nd	nd
Hesperidine	nd	nd	nd	nd
Neohesperidin	nd	nd	nd	nd
Kaemperol-3-glucoside	nd	0.262.	0.359	nd
Fisetin	nd	nd	nd	nd
Baicalin	nd	nd	nd	nd
Ellagic acid	nd	nd	nd	nd
Quercetin	nd	0.199	0.352	nd
Naringenin	nd	0.003	0.013	nd
Silibinin	nd	nd	nd	nd
Hesperetin	nd	0.060	0.051	nd
Morin	nd	nd	nd	nd
Kaempferol	nd	nd	nd	nd
Chrysin	0.439	0.246	0.027	nd
Flavon	nd	1.093	1.716	2.180
Diosgenin	nd	nd	nd	nd
Naphthodianthrone				
Hypericine	nd	nd	nd	nd
Pseudohypericine	nd	nd	nd	nd
Hyperforin	nd	nd	nd	nd

nd, not determined.

Total Bioactive Contents

The achieved results of total phenolic (TPC), flavonoid (TFC) and flavanol (TFLC) contents are given in Table 2. *H. heterophyllum* extracts have displayed incredible variability in the levels of polyphenols associated with the polar of solvents. With increasing polarity index of the solvents, accumulation of phenolics reached to its maximum concentration (132.7 mg GAE/g extract). Methanol extract was found to contain the highest

amount of phenolic compounds followed by acetone, ethanol and chloroform However, many researchers reported that the ethyl acetate solvent, which has a lower polarity index, exhibited higher phenolics in all *Hypericum* species and their plant parts tested than other solvents [42]. Unlike TPC findings, the acetone extract (61.8 mg QE/g extract) were possessed the highest value of flavonoids. Total amount of flavanols ranged between 0.72 mg CE/g extract (in chloroform extract) to 0.89 mg CE/g extract (in acetone extract). Similar trend of association between TPC, TFC and TFLC was observed in the chloroform and ethanol extracts, which had relatively poor contents, respectively.

Indeed, variation of phenolic, flavonoid and flavanol contents in the various solvent causes by many factors concerning solvent, botanical source and bioactive compounds, including solubility, mass transfer, diffusion and osmosis capacities of bioactive compounds from cells [43], affinity between solvent polarity and bioactive compound [44].

Table 2. Total bioactive contents of various organic extracts from *H. heterophyllum* leafs

Total bioactive contents	**Solvents**			
	Methanol	**Ethanol**	**Acetone**	**Chloroform**
Total phenolic content (mg GAE/g extract)	132.7±2.1^{a}	46.1±1.1^{c}	90.6±0.6^{b}	31.5±0.5^{d}
Total flavonoid content (mg QE/g extract)	45.2±1.3^{b}	29.2±1.3^{c}	61.8±1.3^{a}	17.9±1.8^{d}
Total flavanol content (mg CE/g extract)	0.79^{b}	0.73^{c}	0.89^{a}	0.72^{c}

The results are expressed as means ± SD (n = 4). Statistically, each lines was evaluated separately and the differences were shown in lower case according to the $p < 0.05$ level.

Radical Scavenging Activities

Botanical polyphenols exhibits significant biological activities such as antioxidant, anticancer, enzyme, alpha-glucosidase inhibitors and others. Among the polyphenols, flavonoids are especially important and can act as antioxidants due to their ability to scavenge free radicals and chelate transition metals [42]. Also, many scientists had emphasized that solvent polarity can have a strong effect on antioxidants because it was effective on the extraction of plant compounds [45, 46]. A large number of scientific studies revealed strong antioxidant properties of various solvent extracts of *Hypericum* species [3, 6, 42], whereas there is not enough information about that of *H. heterophyllum*, an endemic for Türkiye. In this study, the antioxidant capacities of various solvent extracts of *H. heterophyllum* leaf was comprehensively investigated by five in vitro models of screening, namely, DPPH, ABTS, H_2O_2 and superoxide radical scavenging methods as well as metal chelating activity. The findings obtained from these assays were given in Table 3.

As far as radical scavenging property of various solvent extracts from *H. heterophyllum* leaf is concerned, methanol extract showed the highest DPPH activity (24.9 mg TE/g), while chloroform extract had the lowest activity (6.4 mg TE/g). Interestingly, DPPH activity of *H. heterophyllum* was increased with increasing polarity index of solvents used for extraction. But, in this study, no similar trend was obtained in the other radical scavenging assays. Ethanol extract had the highest radical scavenging with a ABTS value of 59.8 mg TE/g, followed by acetone extract (35.6mg TE/g), methanol extract (33.5 mg TE/g) and chloroform extract (14.1 mg TE/g). However, all solvent extracts from *H. heterophyllum* possessed a lower antioxidant ability to react with DPPH and ABTS radical scavenging compared to the synthetic reference antioxidant BHT (48.9 mg TE/g and 92.8 mg TE/g, respectively). Similarly, Zorzetto and coauthors [6], noted that extracts of *Hypericum reflexum*, *Hypericum canariense* and *Hypericum grandifolium* species displayed the lower DPPH and ABTS activities than trolox standard. Zheleva-Dimitrova and coauthors [47] examined the DPPH and ABTS activities of thirteen *Hypericum* species and noted that while some were lower compared to the standard BHT, most exhibited high activity.

The hydroxyl and superoxide radical scavenging assays for various solvent extracts of *H. heterophyllum* leafs were identified for the first time in this study. As shown in Table 3, the acetone extract exhibited the strongest effect for both activities (351.5 mg AAE/g and 197.3 mg TE/g, respectively), and statistically different from other solvents, whereas the ethanol extract had the lowest effect on these assays. Hunt and coauthors [48] reported that *H. perforatum* had strong inhibitory effect for superoxide radical, and was attributed this radical scavenging assay to hypericin. However, this study was found that hypericin contents of all solvent extracts could not be identified in this study. On the other hand, Şerbetçi and coauthors [49] reported that the superoxide radical scavenging activity and the flavonoid content showed a strong correlation in *Hypericum lydium* extracts.

Table 3. Radical scavenging activities and metal chelating activity of various organic extracts from *H. heterophyllum* leafs

Antioxidant activities	Solvents				BHT
	Methanol	Ethanol	Acetone	Chloroform	
DPPH radical scavenging activity (mg TE/g)	24.9±0.5^{a}	6.6±0.6^{c}	16.6±0.8^{b}	6.4±0.1^{c}	48.9±0.1
ABTS radical scavenging activity (mg TE/g)	33.5±2.3^{b}	59.8±2.0^{a}	35.6±1.3^{b}	14.1±2.4^{c}	92.8±1.5
Hydroxyl radical scavenging activity (mg AAE/g)	232.4±1.8^{b}	98.8±8.9^{c}	351.5±2.7^{a}	228.9±4.2^{b}	nd
Superoxide radical scavenging activity (mg TE/g)	nd	79.7±2.0^{c}	197.3±8.5^{a}	118.3±6.1^{b}	nd
Metal chelating activity (mg EDTAE/g)	nd	6.1±0.6^{c}	15.8±1.1^{b}	26.4±1.0^{a}	nd

nd, not identified. The results are expressed as means ± SD (n = 4). Statistically, each line was evaluated separately, and the differences were shown in lower case according to the $p < 0.05$ level.

Metal Chelating Activities

Metal chelating activity was analysed to determine the potential of the extracts for transition metal ions (Fe^{2+}) to catalyse electron transport, promote free radical scavenge, and protect against many diseases. All the extracts displayed the ability to chelate iron (II) ions except methanol extract. The chelating power of the chloroform extract (26.4 mg EDTAs/g) was higher as compared to the other extracts, while the ethanol extract (6.1 mg EDTAs/g) showed the lowest metal chelating activity. The results were in accordance with Pavithra and Vadivukkarasi [50] who reported that the chloroform extract of *Kedrostis foetidissima* leaf had a higher chelating effect than acetone extract. However, some scientists noted that methanol extracts of many *Hypericum* species had high metal chelating activity [51, 52]. This variation can be attributed to the different phytochemical contents of the species.

Enzyme Inhibitory Activity

When the activity of AChE is increased, it causes damage to the cholinergic system and neurodegenerative diseases occur as a result. Inhibition of AChE is among the important treatment strategies to reduce the progression of Alzheimer's, is a neurodegenerative disease [19]. In the study, the inhibitory effects of *H. heterophyllum* leaf on AChE were evaluated. The inhibitory effects of methanol, ethanol, acetone and chloroform extracts of *H. heterophyllum* leaf on AChE were determined with IC_{50} of 16.32, 13.99, 11.07 and 12.33 µg /ml, respectively.

In silico docking study

We conducted docking studies to reveal the affinity potential of the phytochemicals in the extract against the enzyme. The docking calculations of compounds were presented in Table 1. Quercetin-7-O-glucoside, Diosgenin, and Quercetin-3-glucoside have a stronger MolDock Score than the reference ligand Donepezil. Our docking results have indicated that extracts contain compounds with a high affinity against the acetylcholinesterase enzyme.

The region selected for docking, the placement of the molecules in the active region, and the overlap of the compounds with the reference molecule is shown in Figure 2. It is seen that compounds placed the same active region as Donepezil. These results show that molecules can strongly interact with the active site.

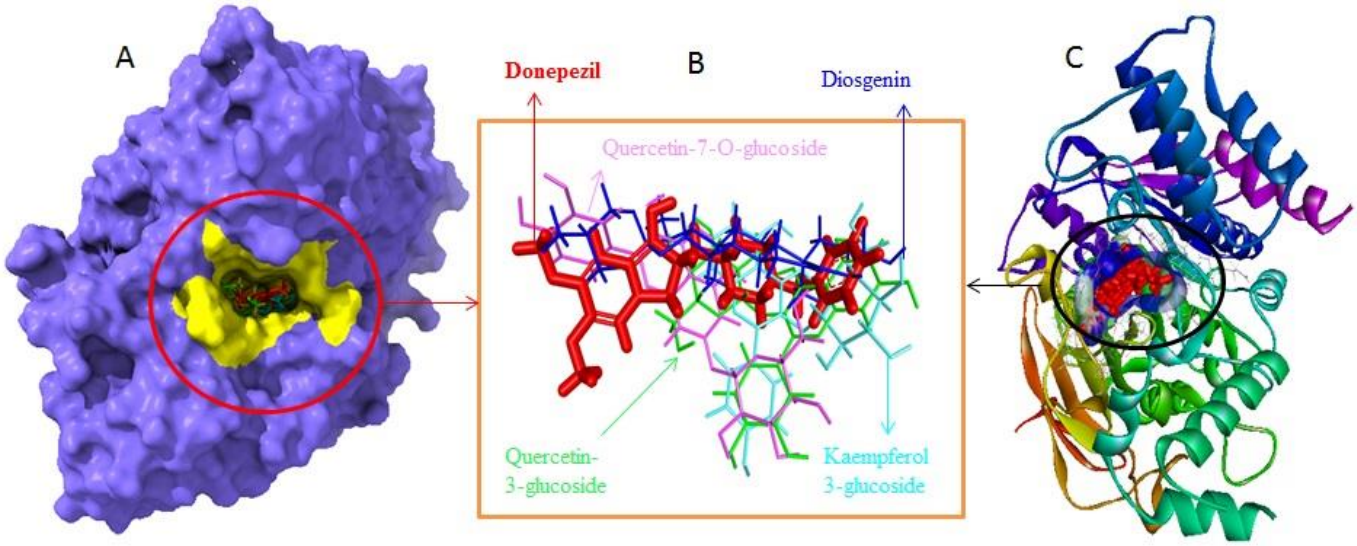

Figure 2. Four phytochemicals at the active region of the enzyme acetylcholinesterase. A: Docking cavity, B:Overlapping of molecules with reference ligand, C: Enzyme and molecules complex

The best 2D interaction maps of the Quercetin-7-O-glucoside, Diosgenin, Quercetin-3-glucoside, and Kaempferol 3-glucoside with the active site of the acetylcholinesterase exhibited in Figure 3. Quercetin-7-O-glucoside showed high binding energy of -164.427 MolDock Score at the active site of the acetylcholinesterase enzyme. In a previous study, the inhibitory effect of Quercetin-7-O-glucoside against the AChE enzyme was reported [53]. The in silico results are in agreement with the experimentally obtained results. The phenol-bearing alpha protons of the catechol of the same compound exhibited two hydrogen bonds with the amino acid residues Ser293 and Arg296. It was seen that Trp86, Tyr72, and Phe 295 found in the binding pocket of the enzyme contributed to hydrogen bond interacting. Likewise, carbon-hydrogen bond interactions with residues Asn87, Ser125, and Phe338 were also observed. As shown in Figure 1, Gly126, Leu130, Gly121, Tyr337, Phe297, Leu289, Asp74, Val73, Pro88, Gln71, and residues were involved in the van der Walls interactions with the entire quercetin-7-O-glucoside, while Tyr341 and Trp286 showed Pi-Pi Stacked interaction with it. Active site residue Tyr124 participated in Pi-lone pair interaction.

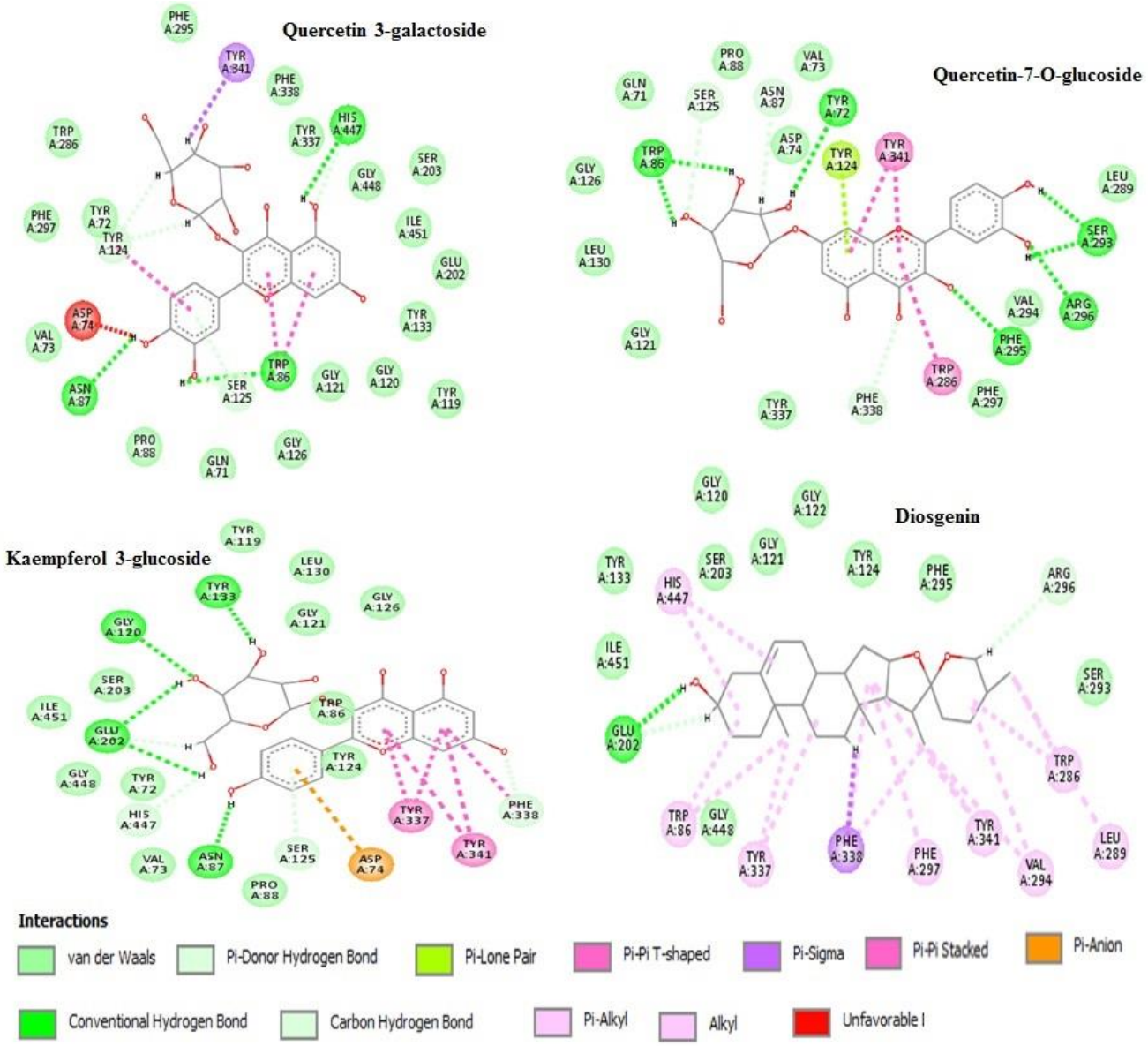

Figure 3. Intermolecular interactions of four compounds with AChE in 2D interactions maps.

Table 4. Docking results of phytochemicals against acetylcholine esterase

Name	PubChem CID	MolDock Score	Rerank Score	HBond
Donepezil (Reference ligand)	3152	-156.942	-133.222	-2.5
Quercimeritrin (Quercetin-7-O-glucoside)	5282160	-164.427	-138.732	-19,6132
Diosgenin	99474	-163.851	-112.678	-5
Quercetin-3-glucoside	5280804	-157.499	-142.065	-12,48
Kaempferol 3-glucoside	5282102	-155.667	-136.084	-14,1825
Hyperoside (Quercetin 3-galactoside)	5281643	-151.287	-136.551	-11.995
Chlorogenic acid	1794427	-136.677	-119.569	-15,3796
Quercetin	5280343	-127.832	-118.166	-14,0382
Catechin	9064	-127.625	-109.786	-11,4117
Epicatechin	72276	-121.828	-60.938	-13.122
Vanillic	8468	-77.134	-69,4839	-5,1063
Sinapinic acid	637775	-95,5217	-83,7354	-2,99805
Protocatechuic acid	72	-73,3194	-62,4955	-3,84256

CONCLUSION

Phytochemical investigation of *H. heterophyllum* leaf extracts presented that this plant could be used in food and pharmaceutical industry. Moreover, *H. heterophyllum* leaf can be used in drug development process. It could play a key role in the treatment of diseases caused by free radicals. The acetone extract exhibited the best AChE inhibition activity. So, acetone extract of this plant leaf could be acetylcholinesterase inhibitors. Due to the major product of chlorogenic acid in ethanol and acetone extracts, *H. heterophyllum* leaf could be the source of chlorogenic acid which displays a large variety of biological effects. Further studies of this plant such as invivo should be carried out to reveal its potential drug candidate.

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