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Biological activities of methanolic extracts from leaves of *Rosa micrantha, Rosa pisiformis, Rosa phoenicia* and *Rosa pulverulenta* species growing in Turkey

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ABSTRACT: The genus *Rosa* L. is represented by 26 species in Turkey and some of them are long been used in the folk medicine due to their potential curative activities. Some of these traditional and folk usages such as antioxidant and antihypertensive agent were evaluated in this study, showing the potential medicinal use of the rose leaves. In our previous studies it was reported that one of the beneficial effects of *Rosa agrestis*, *Rosa sempervirens* and *Rosa horrida* leaves extracts is the antioxidant and free radical scavenging activity of the phenolic and flavonoid contents. Phytochemical analysis of rose leaves extracts indicated the presence of major phytocompounds, including flavonoids like rutin, kaempherol, quercetin and ellagic acid. In order to complement our previous studies on rose species, the methanolic extracts prepared from leaves of *Rosa micrantha*, *Rosa pisiformis*, *Rosa phoenicia* and *Rosa pulverulenta* were tested in vitro for their polyphenolic contents, antioxidant and inhibitory activities against acetylcholinesterase (AChE), buthylcholinesterase (BChE) and angiotensin converting enzyme (ACE) in order to exploit the possible role of these plants as a source of the alternative agents for the prevention and treatment of neurodegenerative disease and hypertension. The results showed that the extracts have potent inhibitory activities against AChE, BChE and ACE, contain high amount of phenolic compounds and possess significant antioxidant activity which may be attributed to a strong reducing power, $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and superoxide radicals scavenging and lipid peroxidation inhibitory activities. The enzyme kinetic studies revealed that ellagic acid, kaempherol and quercetin inhibit reversibly both the AChE and BChE enzymes in non-competitive manner. The results showed that the leaves of rose species under study could provide new therapeutical applications.

KEYWORDS: Rosa species; leaves; phenolic compounds; antioxidant; anticholinergic activity; angiotensin converting enzyme inhibition

1. INTRODUCTION

Rose species are medicinal herbs with wide applications in the food, cosmetics, and pharmaceutical industries. Many studies reported the antioxidant, anti-hypotensive and anti-inflammatory therapeutic activities of the different rose parts [1]. The rose leaves were reported to be a rich source of phenolic compounds and useful for treating diarrhea, nephritis, wounds and burns [2,3]. It was reported that one of the benefficial effects of *Rosa agrestis* [4], *Rosa sempervirens* [5,6], *Rosa horrida* [7], *Rosa canina* L., *Rosa moschata* Herrm. [5] and *Rosa roxburghii* Tratt. [2] leaves is conferred to antioxidant properties of phenolic compounds, which have been related to their capability to act as radical scavengers, hydrogen and electron donors and reducing agents. Phytochemical analysis of *R. agrestis*, *R. semperverens* and *Rosa roxburghii* leaves extracts indicated the presence of flavonoids like diosmetin, astragalin, quercitrin, quercetin, hyperoside, kaempherol, (+)-catechin and myricetin [2,4,6]. Nowak and Gawlik-Dziki [3] evaluated phenolic content and antioxidant activity of the leaves extracts prepared from 17 rose species of Poland flora and reported that all of the examined leaves extracts show potent antioxidant properties which correlated with the amount of phenolic compounds. Ellagic acid, quercetin and kaempferol contents of these species were detected as the major flavonoids using SPE-RP-HPLC methods.

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The findings that reactive oxygen species (ROS)-mediated oxidative damage may play significant role in various pathological disorders have significantly increased the interest in herbal medicines as a a possible source of free radical scavengers and importance of identifying new targets for the development of effective therapeutic strategies to manage diseases, such as Alzheimer's disease (AD), cardiovascular disorders, and cancer [1]. The flavonoids might be effective at treating deficits in cognition and important for the development of drug candidates for the symptomatic treatment of Alzheimer's disease (AD). ACE inhibitors of plant-origin are being evaluated as therapeutic agents for the prevention and treatment of hypertension which increases the risk of developing cardiovascular disease [1]. In this context, the rose species may have played an important role as a source of new therapeutic agents. Due to the limited researches concerning the biological acitivites of rose species leaves extracts, the aim of this study was to assess the antioxidant and inhibitory activities towards AChE, BChE and ACE in order to highlight the potential benefits of this plant species in the patgogenesis of hypertension, ischemia/reperfusion injury, inflammation, cancer and neurodegenerative diseases.

2. RESULTS and DISCUSSION

2.1. Total phenolic compound and flavonoid contents

The present study evaluated the antioxidant, anti-cholinergic and ACE inhibitory effects of the leaves of *R. micrantha, R. phoenicia, R. pisiformis* and *R. pulverulenta*. Studies on the antioxidant potential of *R. pulverulenta* [8], *R. micrantha* [9] and *R. pisiformis* [10] hips have been conducted, but no research has been done on the potential health benefits of their leaves. Strong antioxidant effects of polyphenols and terpenoids contained in the different parts of rose species have been demonstrated in a review article [1]. For this reason, it is becoming increasingly important to use the extraction products obtained from rose species as a natural antioxidant source in phytotherapy health products.

The extractable compounds, total phenolic compounds and flavonoids in leaves methanolic extracts of four rose species were shown in Table 1. The extractable compounds contents ranged from 74.86 to 124.84 mg/g (7.4 % to 12.4%) dry plant material. Among the four extracts, the extract of *R. phoenicia* contained the largest amount of extractable compounds, followed by *R. pulverulenta* and *R. micrantha*, while the extract of *R. pisiformis* contained the smallest one. Although the extraction yield of total extractable compounds of *R. micrantha* and *R. pisiformis* extracts were the smallest, the total phenol/extractable compounds ratio were comparable with that found for the extracts of *R. phoenicia* and *R. pulverulenta*.

Table 1. Total extractable compounds (EC), total phenolic compounds (PC) and flavonoids in *R. micrantha*, *R. pisiformis*, *Rosa phoenicia* and *R. pulverulenta* leaves methanolic extracts.

Taxon	EC (mg/g DW)	PC (mg GAE/g extract)	PC (mg GAE/g DW)	Flavonoids (mg CE/g extract)	Flavonoids (mg/g DW)	PC/EC (%)
R. micrantha	82.03	212.98 ± 7.59^{a}	17.47 ± 0.56^{a}	84.91 ± 5.51^{a}	8.64 ± 0,51^{a}	21.29
R. pisiformis	74.86	214.13 ± 5.55^{a}	16.05 ± 0.41^{a}	86.05 ± 1.07^{a}	7.99 ± 0,26^{a}	21.44
R. phoenicia	124.84	222.90 ± 5.79^{a}	27.63 ± 0.71^{b}	102.75 ± 2.40^{b}	12.98 ± 0,72^{b}	22.13
R. pulverulenta	103.26	227.82 ± 3.91^{a}	23.46 ± 0.41^{c}	96.12 ± 1.35^{c}	10.44 ± 0,58^{c}	22.71

Values were the means ± SD of three replicates. Different values in the same column indicate the significant differences ($p < 0.05$) between the samples.

The total phenolic content per gram crude extract of methanol extracts were found to be similar (p>0.05). As the methanol extraction resulted in lowest yield of extractable compounds of *R. micrantha* and *R. pisiformis* leaves, the content of phenolic compounds and flavonoids per gram of dry weight (DW) in extracts of *R. phenicia* and *R. pulverulenta* leaves were larger than the content obtained in the extracts of *R. micrantha* and *R. pisiformis* leaves. These contents were smaller compared to those reported for *R. agresitis* (489.76 mg GAE/g exract) [4] and *Rosa roxburghii* (167.48–190.14 mg GAE/g DW) [2]. Similar results were observed by Ozsoy et al. [7] for *R. horrida* leaves (21.9 mg/ g DW) and Ouerghemmi et al. [5] for *R. sempervirens* (217 mg/g extract) leaves. The phenolic compounds contents found in the extracts of seventeen rose species leaves were in agreement with our results [3].

Our results pointed out greater phenolics content in leaves compared with hips of *R. micrantha* where the phenolic content ranged from 40.58 mg GAE/g to 142.34 mg GAE/g [9, 11], however lower than that reported for *R. pisiformis* (78 mg/g DW) [10,12] and *R. pulverulenta* (94 mg/g DW) hips [12].

2.2. Antioxidant activity

Antioxidant activities of the extracts under study were presented in Table 2. It was seen that the extracts show similar reducing powers and capability of scavenging DPPH•, ABTS•+ and superoxide radicals and preventing against lipid peroxidative damage, however this efficacy was weaker than that of rutin ($p < 0.05$). DPPH free radicals scavenging ability of the extracts was more significant than their capability to prevent lipid peroxidation propagation and reduce the generation of ABTS•+ and superoxide radicals.

Table 2. Antioxidant capacity levels of the *R. micrantha*, *R. pisiformis*, *R. phoenicia* and *R. pulverulenta* leaves methanolic extracts as estimated by EC_{50}, TEAC and FRAP values.

Extract	EC_{50} (mg/mL)				TEAC value (mM /L Trolox)	FRAP value (mM/L Fe^{2+})
	Anti-LPO	DPPH•	Superoxide•-	ABTS•+		
R. micrantha	0.493 ± 0.003[a]	0.269 ± 0.024[a]	0.546 ± 0.027[a]	0.492 ± 0.01[a]	2.08 ± 0.076[a]	2.54 ± 0.010[a]
R. pisiformis	0.475 ± 0.016[a]	0.272 ± 0.006[a]	0.536 ± 0.018[a]	0.501 ± 0.01[a]	2.13 ± 0.023[a]	2.45 ± 0.080[a]
R. Phoenicia	0.414 ± 0.012[b]	0.229 ± 0.007[b]	0.459 ± 0.002[b]	0.458 ± 0.02[b]	2.11 ± 0.046[a]	2.75 ± 0.005[b]
R. pulverulenta	0.404 ± 0.011[b]	0.227 ± 0.007[b]	0.490 ± 0.008[b]	0.457± 0.07[b]	2.13 ± 0.015[a]	2.62 ± 0.010[b]
Rutin	0.160 ± 0.004[c]	0.169 ± 0.001[c]	0.181 ± 0.002[c]	0.362 ± 0.01[c]	2.15 ± 0.017[a]	3.90 ± 0.015[c]

Values were the means ± SD of three replicates. Different values in the same column indicate the significant differences ($p < 0.05$) between the samples.

The extracts exhibited a high total antioxidant potential expressed as the TEAC and FRAP value at 1.25 mg/mL, which were comparable to that of rutin ($p > 0.05$) at the same concentration.

Since superoxide radical formation and lipid peroxidation occur during the functioning of normal metabolic pathways, the superoxide and peroxyl radicals scavenging potential of the extracts reflect their antioxidant activities in the biological system.The unsaturated bonds of phospholipids in membranes easily react with free radicals and cause lipid peroxidation, a chain reaction that produces aldehydes, which directly damages the membrane structure and indirectly other cell components, leading to the development of various pathological states. For this reason, the activities of leaves extracts of rose species on the inhibition of iron-induced lipid peroxidation in phosphatidylcholine liposomes were investigated. It was demonstrated that each extract has an inhibitory effect on phospholipid degradation. These results showed that even though the examined extracts are not as effective lipid peroxidation inhibitors as rutin, they can stop the chain reaction by donating hydrogen atoms and removing lipid peroxide radicals. These results were consistent with other published papers reporting that rose species are capable to reduce the excessive production of peroxyl and superoxide radicals [4,7,13]. It was concluded that the extracts under study may be used as an effective therapeutic agent for treatment of some pathological conditions associated with oxidative damage [1].

Metal ions induce lipid peroxidation. Fe^{3+} reacts with lipids to decompose alkyl free radicals, hydroperoxides, activating peroxyl and alkoxyl radicals, molecular oxygen to form singlet oxygen. It was observed that the rose leaves extracts examined in this study had the reducing capability of ferric ion. The results were in accordance with those reported for *R. agrestis*, *R. horrida*, and *R. sempervirens* [4-7].

Our findings that rose species can neutralize synthetic radicals such as DPPH• and ABTS•+, used to determine the hydrogen and electron donating effects of the studied extracts in biological systems, were found to be compatible with the literature [3-7].

It was concluded that the extracts under study have the antioxidative potential to inhibit the propagation of the iron-mediated lipid peroxidation, act as the donor for hydrogen atoms and electrons, thus could protect against increased oxidative stress-induced tissue damage, which contributes to the development of hypertention, cancer, neurodegenerative diseases, cardiovascular and diabetic complications such as atherosclerosis.

Rosehip and leaves extracts showed the similar antioxidant profile, wich is strongly correlated with their phenolic content [9-11].

2.3. AChE and BChE inhibitory activities

Pathological and biochemical data obtained in studies on Alzheimer's disease (AD) showed that there is no definitive solution for the treatment of this disease. New approaches have been developed to reduce the decline in brain functions or slow down the development of the disease. One of the treatment approaches for AD is to expand synaptic acetylcholine retention by inhibiting the activity of acetylcholinesterase, which results in enhancement of cholinergic transmission. AChE inhibitors are used as the most effective drug group in the treatment of AD. Previous investigations have reported the increased activity of BChE in the most affected regions in brain of Alzheimer's patients compared to the normal ones, so the inhibition of the BChE has gained importance in the treatment [14]. Many studies have reported that natural or synthesized flavonoid analogs exert neuroprotective effects by inhibiting AChE and BChE enzymes, Aβ fibril formation and tau aggregation. Researchs suggested that some natural flavonoids can exert beneficial effects on neurological processes potentially influencing neuronal signaling pathways [15].

In the present study, methanol extracts prepared from the investigated rose species were tested for their AChE and BChE inhibitory activities. Donepezil chloride [16,17], a reversible selective acetylcholinesterase inhibitor that has been used successfully in the AD treatment was used as a standard.

Inhibitory activities of the extracts and donepezil against AChE and BChE were shown in Table 3. Taking into consideration EC_{50} values, the inhibitory effectiveness of the extracts were found to be very close to each other ($p > 0.05$), but significantly lower than that of donepezil ($p < 0.05$).

Table 3. AChE, BChE and ACE inhibitory activities of the *R. micrantha*, *R. pisiformis*, *Rosa phoenicia* and *R. pulverulenta* leaves methanolic extracts.

	EC_{50} (μg/mL)		
Extract	**AChE inhibition**	**BChE inhibition**	**ACE inhibition**
R. micrantha	31.22 ± 0.25[a]	31.16 ± 0.04[a]	360.5 ± 9.5[a]
R. pisiformis	31.29 ± 0.04[a]	31.13 ± 0.12[a]	340.1 ± 6.9[a]
R. phoenicia	31.14 ± 0.08[a]	30.63 ± 0.25[a]	350.6 ± 7.5[a]
R. pulverulenta	31.25 ± 0.11[a]	31.91 ± 0.32[a]	360.3 ± 8.9[a]
Standard	Donepezil (μg/mL)	Donepezil (μg/mL)	Captopril (μg/mL)
Donepezil	0.14 ± 0.01[b]	0.20 ± 0.03b	0.11 ± 0.01[b]

Values were the means ± SD of three replicates. Values with different letters in the same column were significantly (p <0.05) different.

It was reported that phenolic compounds, especially kaempherol, quercetin and ellagic acid are the major polyphenols of the leaves of rose species [3,4,18]. For this reason, ellagic acid, kaempherol and quercetin were investigated for their inhibitory effects against AChE and BChE using donepezil as a referance compound, in order to determine the enzyme's inhibition type. The results, expressed as Ki and IC_{50} values were summarized in Table 4 and Table 5.

It was found that the compounds under study inhibited AChE and BChE in a reversible non-competitive manner (Figure 1 and Figure 2, respectively).

Table 4. Inhibition type and inhibitory effects of ellagic acid, kaempferol and quercetin on AChE expressed as IC_{50} and Ki values.

Compounds	**IC_{50} (μM)**	**K_i (μM)**	**Type of inhibition**
Ellagic acid	0.37 ± 0.01[a]	0.45 ± 0.03[a]	Non-competitive
Kaempferol	1.15 ± 0.06[b]	1.85 ± 0.05[b]	Non-competitive
Quercetin	1.64 ± 0.08[c]	2.04 ± 0.06[c]	Non-competitive
Donepezil	0.36 ± 0.05[a]	0.40 ± 0.03[a]	Non-competitive

Values were the means ± SD of three replicates. Values with different letters in the same column were significantly different at p<0.05.

Table 5. Inhibition type and inhibitory effects of ellagic acid, kaempferol and quercetin on BChE expressed as IC_{50} and Ki values.

Compounds	**IC_{50} (μM)**	**K_i (μM)**	**Type of inhibition**
Ellagic acid	0.48 ± 0.06[a]	0.44 ± 0.01[a]	Non-competitive
Kaempferol	2.39 ± 0.15[b]	2.26 ± 0.14[b]	Non-competitive
Quercetin	2.24 ± 0.12[b]	2.37 ± 0.08[b]	Non-competitive
Donepezil	0.50 ± 0.05[a]	0.81 ± 0.11[c]	Non-competitif

Values were the means ± SD of three replicates. Values with different letters in the same column were significantly ($p<0.05$) different.

From the IC_{50} values, it was seen that ellagic acid had the highest AChE inhibitory activity, which was comparable to that of donepezil ($p < 0.05$), followed by quercetin, while kaempherol was the least effective AChE inhibitor.

Khan et al. [19] investigated the ability of quercetin, rutin, kempferol 3-O-d-galactoside, and macluracantone to inhibit AChE and BChE *in vitro*. They reported that quercetin show inhibitory potency against AChE (IC_{50}= 353.86 µM/L) and BChE (IC=420.76 µM). In this study, it was observed that quercetin inhibits both enzymes reversibly but at a lower concentration, so it is thought that the leaves of the rose species under study may be a potential drug candidate source, as the flavonoids such as ellagic acid, kaempferol and quercetin reversibly inhibit AChE, as well as BChE.

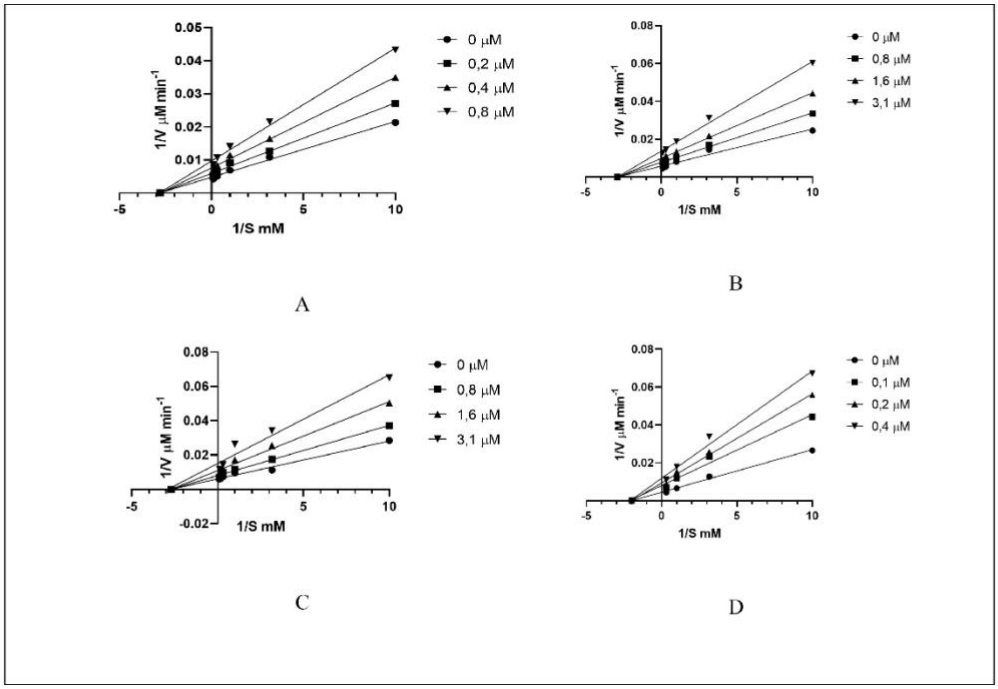

Figure 1. The double reciprocal (Lineweaver-Burk) plot of ellagic acid, kaempherol, quercetin and donepezil against AChE.
A: Ellagic acid; B: Kaempferol; C: Quercetin; D: Donepezil

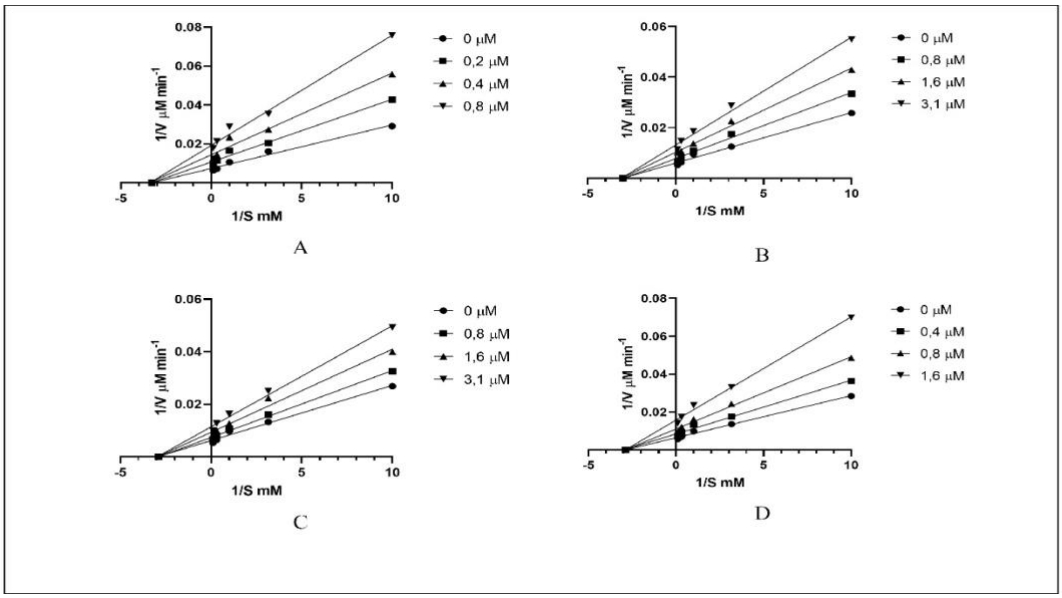

Figure 2. The double reciprocal (Lineweaver-Burk) plot of ellagic acid, kaempherol, quercetin and donepezil against BChE.
A: Ellagic acid; B: Kaempferol; C: Quercetin; D: Donepezil

2.4. ACE inhibitory activity

ACE inhibition is one of the therapeutic approachs of hypertension regulation and therapy. For this reason, extensive investigations have been done to identify natural compounds inhibiting ACE. Previous studies revealed that natural compounds such as peptides [20], triterpenes [21], coumarins [22] can be therapeutic agents targeting ACE inhibition. In many studies, plants rich in polyphenols have been reported to exert hypertension-lowering effects by inhibiting ACE and protect from free radicals mediated oxidative stress, involved in the development of high blood pressure [23,24,25].

Different experiments with spontaneously hypertensive rats have shown that quercetin [26], ferulic acid [27] and tannic acid [28] reduce blood pressure *in vivo*. Guerrero et al. [29] examined the flavonoids structure-ACE inhibitory activity relationship, Al Shukor et al. [30] reported the ACE inhibitory activities of 22 compounds, including quercetin, ellagic acid, kaempferol and rutin and indicated that all the phenolic compounds were able to inhibit ACE, but with different potencies, depending on their structure.

The present study evaluated the inhibition activities of the investigated rose species leaves extracts and captopril, used as drug for hypertension treatment. The results were represented in Table 3.

When the EC_{50} values were compared, it was found that the extracts which showed similar effects as can be seen from the insignificant differences between them ($p > 0.05$) were less active than captopril ($p < 0.05$). It was determined that the rose species examined in this study showed ACE inhibitory activity, and it was concluded that this effect may be due to the flavonoids they contain. Our study was in agreement with above mentioned studies showing that the rose species possess antihypertensive effect.

3. CONCLUSION

The present study results revealed that leaves of *R. micrantha, R. pisiformis, R. phoenicia and R. pulverulenta* have the antioxidant potential, which was related to the presence of phenolic compounds. Considering the importance of plants with reversible inhibitory activity towards AChE and BChE in the development of promising drugs that could change the course of the therapy of neurodegenerative diseases in the future, it was concluded that the leaves of *R. micrantha*, *R. phoenicia*, *R. pulverulenta* and *R. pisiformis* could improve therapeutic outcomes. By examining the inhibitory potential of the extracts against ACE, it was concluded that they can be used as a natural source for hypertension treatment. The results showed that the leaves of *R. micrantha*, *R. phoenicia*, *R. pisiformis* and *R. pulverulenta* exert antioxidant, anticholinergic and anti-hypertensive effects that may be of therapeutic interest because they can play a pivotal role in the discovery and development of new multitargeted drugs for managing neurodegenerative and cardiovascular diseases.

4. MATERIALS AND METHODS

4.1. Plant materials and preparation of the extracts

Rosa phoenicia Boiss.; *Rosa pulverulenta* Bieb.; *Rosa micrantha* Sm. and *Rosa pisiformis* leaves were authenticated by Prof. Dr. Şukran Kültür, and voucher specimens were deposited in Department of Pharmaceutical Botany Herbarium. ISTE numbers and detailed localities were given in Table 6.

Table 6. ISTE numbers and locality of *R. micrantha*, *R. pisiformis*, *R. phoenicia* and *R. pulverulenta* species.

Taxon	İSTE	Locality	Plant material
Rosa micrantha Sm.	68069	Kırklareli	2.60 g
Rosa pisiformis (Christ) D. Sosn	79654	Van	3.35 g
Rosa phoenicia Boiss.	98230	Mersin-Gözne	4.16 g
Rosa pulverulenta Bieb.	72024	Balıkesir-Kaz mountains	16.22 g

Plant samples were dried at room temperature. The dried leaves were manually ground to a fine powder and extracted with 500 ml methanol and refluxed for 5 hours in a Soxhlet apparatus. The methanolic extracts were evaporated under vacuum at 40° to 50°C using a rotary evaporator. Extracts were stored at -20°C until the use.

4.2. Determination of total phenolic compound and flavonoid contents

The total phenolic content of the extracts was analysed by using Folin-Ciocalteu's reagent and expressed as gallic acid equivalents (GAE) [31]. The flavonoid content was determined according to the $AlCl_3$ method and expressed as catechin equivalents (CA) [32].

4.3. Antioxidant activity

Antioxidant activity was estimated using TBA [33] based on the lipid peroxidation (LPO) of liposomes [34], Trolox equivalent antioxidant coefficient (TEAC) [35], expressed as trolox equivalents and Ferric ion reducing antioxidant power (FRAP) expressed as ferrous ions equivalents [36], DPPH free [37] and superoxide anion radical scavenging activities assays [38].

4.4. AChE and BChE inhibitory activities

AChE and BChE inhibitory activities of the tested extracts were estimated using Ellman's method [39]. In order to determine the inhibition type, AChE and BChE inhibitory activities of three concentrations of kaempherol, quercetin and ellagic acid obtained from Sigma Aldrich (0.5; 0.25; 0.125 and 0.0625 mM/mL to produce in reaction mixture final concentrations of 3.1; 1.6; 0.8 and 0.4 µM/mL) were evauated by measuring the effects of increasing concentrations of substrates (acetylthiocholineiodide or butyrylthiocholineiodide) (10 mM to 0.1 mM) or donepezil, a reversible selective cholinesterase inhibitor used as a standard (0.0625; 0.031; 0.016 and 0.008 mM/mL or 0.026; 0.013; 0.0065 and 0.00325 mg/mL to produce in reaction mixture final concentrations of 1,6; 0,8; 0,4; 0.2 and 0.1 µM/mL or 0.32; 0.16; 0.08 and 0.04 µg/mL). 1 units of AChE and 5 units of BChE were used for both AChE and BChE activity assays. Inhibitory constants (Ki) were determined by construction of 1/V versus 1/[S]) (Lineweaver Burk) plots.

4.5. ACE inhibitory activity

The inhibitory activity of the extracts against ACE (40; 20; 10 and 5 mg/mL to produce in reaction mixture final concentrations of 1.8; 0,9; 0,45 and 0.225 mg/mL) or captopril, used as a standard (0,01; 0,005; 0,0025 and 0,00125 mg/L to produce in reaction mixture final concentrations of 0.45; 0.225; 0.112 and 0.056 µg/mL), was evaluated by kinetic spectrophotometric method based on the chemical reaction between ACE and furylacryloylphenylalanyl-glycyl-glycine (FAPGG) as the substrate according to Ali et al [22].

5. STATISTICAL ANALYSIS

Statistical significance analysis was done by one way ANOVA test. Determination of enzyme kinetic parameters and calculations were performed using GraphPad Prism version 9.00 (GraphPad Software Inc., San Diego, CA, USA). Results were considered statistically significant at $p < 0.05$.

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