



In Vitro Protection by *Crataegus microphylla* Extracts Against Oxidative Damage and Enzyme Inhibition Effects

Crataegus microphylla Ekstrelerinin Oksidatif Hasara Karşı *In Vitro* Koruma ve Enzim İnhibisyonu Etkileri

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ABSTRACT

Objectives: *Crataegus* species have been used as food and also in folk medicine for the treatment of various diseases. The present study aimed to make investigations on the biologic properties of different extracts prepared from *Crataegus microphylla* C. Koch, which was collected from Turkey.

Materials and Methods: Dried leaf, stem bark, and fresh fruit samples of *C. microphylla* were separated and ethanol extract, acidified (0.5% HCl, pH: 2.5) ethanol extract, ethanol:water (1:1) extract, methanol extract, acidified (0.5% HCl, pH: 2.5) methanol extract, methanol:water (1:1) extract, water extract, and acidified (0.5% HCl, pH: 2.5) water extract were prepared for each. Various biologic effects such as the prevention of oxidative DNA damage, acetylcholinesterase, tyrosinase, α -glucosidase inhibition, and antioxidant effects with 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, PRAP, and FRAP assays of these extracts at different concentrations were studied.

Results: Acidified methanol extract of stem barks exhibited the highest acetylcholinesterase and tyrosinase inhibitions among the other extracts with IC_{50} values of $204.02 \pm 0.95 \mu\text{g/mL}$ and $37.30 \pm 0.27 \mu\text{g/mL}$, respectively. Acidified ethanol extract of leaves was the most efficient extract against α -glucosidase, giving an IC_{50} of $15.78 \pm 0.14 \mu\text{g/mL}$. The IC_{50} value of the acidified ethanol extract for DPPH was $9.89 \pm 0.09 \mu\text{g/mL}$. Methanol extracts of leaves and stem barks at the dose of $125 \mu\text{g/mL}$ exhibited significant protective activity against DNA strand scission by hydroxyl radicals ($\cdot\text{OH}$) on supercoiled pBR322 DNA.

Conclusion: Acidified methanol or ethanol extracts prepared with stem bark and leaf from *C. microphylla* have potential antioxidant, hypoglycemic, and neuroprotective effects.

Key words: DPPH, FRAP, hawthorn, PRAP, Rosaceae

ÖZ

Amaç: *Crataegus* türleri gıda olarak ve halk arasında çeşitli hastalıkların tedavisinde kullanılmaktadır. Bu çalışma Türkiye'den toplanan *Crataegus microphylla* C. Koch'tan hazırlanan farklı ekstraktların biyolojik özelliklerini araştırmayı amaçlamaktadır.

Gereç ve Yöntemler: Kurutulmuş yaprak, gövde kabuğu ve taze meyve örnekleri ayrıldı ve etanol ekstresi, asitlendirilmiş (%0.5 HCl, pH: 2.5) etanol ekstresi, etanol:su (1:1) ekstresi, metanol ekstresi, asitlendirilmiş (%0.5 HCl, pH: 2.5) metanol ekstresi, metanol:su (1:1) ekstresi, su ekstresi ve asitlendirilmiş (%0.5 HCl, pH: 2.5) su ekstresi hazırlandı. Ekstrelerin oksidatif DNA hasarının önlenmesi, asetilkolinesteraz, tirozinaz, α -glukozidaz inhibisyonu ve antioksidan aktivitesi 2,2-difenil-1-pikrilhidrazil (DPPH) radikal süpürme, PRAP ve FRAP gibi çeşitli biyolojik aktiviteleri farklı konsantrasyonlarda araştırılmıştır.

Bulgular: Gövde kabuklarından asitlendirilmiş metanol ekstraktı sırasıyla $204.02 \pm 0.95 \mu\text{g/mL}$ ve $37.30 \pm 0.27 \mu\text{g/mL}$ IC_{50} değerleri ile diğer ekstreler arasında en yüksek asetilkolinesteraz ve tirozinaz inhibisyonu göstermiştir. Yaprakların asitlendirilmiş etanol ekstresi, α -glukozidaz enzimine karşı $15.78 \pm 0.14 \mu\text{g/mL}$ ile en düşük IC_{50} değerini göstermiştir. DPPH için asitlendirilmiş etanol ekstraktının IC_{50} değeri $9.89 \pm 0.09 \mu\text{g/mL}$ bulunmuştur. $125 \mu\text{g/mL}$ dozunda yaprakların ve gövde kabuklarının metanol ekstreleri, süpersarmal pBR322 DNA üzerinde hidroksil iyonu ($\cdot\text{OH}$) tarafından DNA sarmalının kesilmesine karşı önemli koruma aktivitesi sergiledi.

Sonuç: *C. microphylla* gövde kabuğu ve yaprak ile hazırlanan asitlendirilmiş metanol veya etanol ekstrelerinin potansiyel antioksidan, hipoglisemik ve nöroprotektif etkileri bulunmaktadır.

Anahtar kelimeler: DPPH, FRAP, alıç, PRAP, Rosaceae

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INTRODUCTION

The *Crataegus* genus (Rosaceae) has approximately 200 species worldwide and 24 species in Turkey.^{1,2} All plant species in this genus have the common name "Hawthorn".³ *Crataegus microphylla* C. Koch is one of the wild edible fruits in Turkey.⁴ *Crataegus* species have been used as food and also in folk medicine for the treatment of different heart diseases and diabetes for hundreds of years.^{3,5,6} Fruits of the *Crataegus* species are used for stimulating digestion, improving blood circulation, and for the treatment of diarrhea, abdominal pain, amenorrhea, hypertension, and hyperlipidemia in Chinese traditional medicine.³ In addition, products that include the extracts of some *Crataegus* species are consumed as natural health products in Europe, Asia, and North America.^{7,8} Epidemiologic studies and associated meta-analyses showed that long-term consumption of plant polyphenols in diet protected against the development of cancers, cardiovascular diseases, diabetes, osteoporosis, and neurodegenerative diseases.⁹⁻¹³

In addition to its ethnopharmacologic use, the preventive effect of *C. microphylla* fruit extract against genotoxicity induced by methyl methanesulfonate has been investigated in human cultured blood lymphocytes and found to reduce the oxidative stress and genotoxicity induced by toxic compounds. This activity is attributed to its phenolic content and antioxidant potential.¹⁴

By the results of many pharmacologic studies performed with extracts and isolated constituents of *Crataegus* species, flavonoids and proanthocyanidins were found to be responsible for the cardiovascular protective activity of the plant.⁸ With phytochemical studies, D-sorbitol, apigenin, naringenin, eriodictoyl, vitexin, vitexin-4'-*O*-rhamnoside, hesperetin, luteolin, luteolin 7-*O*-glucoside, quercetin, and hyperoside have been isolated from *C. microphylla*.¹⁵⁻¹⁸ Hyperoside was found to be the major compound in leaves and flowers of *C. microphylla*.¹⁷

Oxidative stress is involved in several neurodegenerative disease and degenerative disorders such as cancer, arteriosclerosis, and diabetes.¹⁹ As the accepted consent, the phenolic content determines the antioxidative properties of plant species, and polyphenols play a role in the prevention of chronic human diseases.⁹ The prevention of DNA damage, antioxidant activity, and total phenolic and flavonoid contents of extracts of new sources are very important in explaining their biochemical properties and behavior. In particular, studies of inhibition of these enzymes and prevention of DNA oxidative damage will also enlighten researchers to perform further studies in terms of neurodegenerative enzyme inhibition, anti-diabetic activity, and preventing the conversion to mutagenic forms with various extracts from *C. microphylla*.

In this study, prevention of oxidative DNA damage, acetylcholinesterase (AChE), tyrosinase, α -glucosidase inhibition behaviours and antioxidant effects: 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging effect, phosphomolybdenum-reducing antioxidant power (PRAP), ferric-reducing antioxidant power (FRAP) with total phenolic and total flavonoid contents of the *C. microphylla* leaves, stem barks and fruits that were extracted with ethanol, methanol, and water were investigated. The biologic evaluation of the aerial part extracts of *C. microphylla* was investigated for the first time in this work.

EXPERIMENTAL

Plant material and sample preparation

Leaf, stem bark (B), and fruit of *C. microphylla* were collected from Kale, Gümüşhane-Turkey, in September 2015. A voucher specimen was deposited at the Hacettepe University, Faculty of Pharmacy, Herbarium (Voucher No: HUEF 15021).

Dried leaf (L), B and fresh fruit (F) samples of *C. microphylla* were separated and 50 g of L, B, and F was extracted with 250 mL of various solvents to obtain ethanol extract (1), acidified (0.5% HCl, pH: 2.5) ethanol extract (2), ethanol:water (1:1) extract (3), methanol extract (4), acidified (0.5% HCl, pH: 2.5) methanol extract (5), methanol:water (1:1) extract (6), water extract (7), and acidified (0.5% HCl, pH: 2.5) water extract (8) for each, respectively. Extractions were performed in a shaker for 4 h x 3 times, for each sample. Extracts were filtered and evaporated under reduced pressure using a rotary evaporator. Crude extracts were kept in a refrigerator at +4°C until used. All of the extracts in Table 1 were tested in all assays.

Table 1. The codes and yields (w/w) of the extracts prepared with various solvents of leaf, bark and fruit from *C. microphylla*

	Codes	Yields (w/w)
Leaf in EtOH	L1	16.2
Leaf in EtOH, pH 2.5	L2	15.8
Leaf in EtOH:H ₂ O (1:1)	L3	23.2
Leaf in MeOH	L4	20.6
Leaf in MeOH, pH 2.5	L5	17.6
Leaf in MeOH:H ₂ O (1:1)	L6	24.6
Leaf in H ₂ O	L7	10.2
Leaf in H ₂ O, pH 2.5	L8	18.2
Bark in EtOH	B1	5.8
Bark in EtOH, pH 2.5	B2	7.4
Bark in EtOH:H ₂ O (1:1)	B3	9.1
Bark in MeOH	B4	10.0
Bark in MeOH, pH 2.5	B5	8.3
Bark in MeOH:H ₂ O (1:1)	B6	9.6
Bark in H ₂ O	B7	7.3
Bark in H ₂ O, pH 2.5	B8	6.8
Fruit in EtOH	F1	10.9
Fruit in EtOH, pH 2.5	F2	10.0
Fruit in EtOH:H ₂ O (1:1)	F3	6.3
Fruit in MeOH	F4	10.7
Fruit in MeOH, pH 2.5	F5	9.8
Fruit in MeOH:H ₂ O (1:1)	F6	5.7
Fruit in H ₂ O	F7	8.6
Fruit in H ₂ O, pH 2.5	F8	9.5

*L: Leaf, B: Bark, F: Fruit, w/w: Weight per weight

Enzyme inhibitions

Acetylcholinesterase inhibition

AChE inhibition was examined using the method described by Ellman et al.²⁰ and Ingkaninan et al.²¹ Galantamine was used as the positive control. All extracts (L1-8, B1-8 and F1-8) at various concentrations were separately added to a 96-well microplate and incubated for 15 min at 25°C. Absorbance was measured at 412 nm using a 96-well microplate reader. Inhibition of AChE was calculated using Formula 1, in which A_{control} is the activity of enzyme without extract (solvent in buffer pH=8) and A_{sample} is the activity of enzyme with extract at various concentrations. The inhibitory concentrations of 50% of AChE (IC_{50}) values were calculated from the graph of the percentage inhibition against extract concentrations.

$$\text{Formula 1. Inhibition (\%)} = \left[\frac{A_{\text{sample}} - A_{\text{control}}}{A_{\text{control}}} \right] \times 100$$

Tyrosinase inhibition

Tyrosinase inhibition was examined using the method described by Masuda et al.²² Kojic acid was used as the positive control. The tyrosinase inhibition percentage of all extracts (L1-8, B1-8 and F1-8) (20 μ L) at various concentrations was calculated using Formula 1. The inhibitory concentration of 50% of tyrosinase (IC_{50}) values was calculated from the graph of the percentage inhibition against extract concentrations.

α -glucosidase inhibition

α -glucosidase inhibition was examined using the method described by da Silva Pinto et al.²³ Acarbose was used as the reference drug. The α -glucosidase inhibition percentage of all extracts (L1-8, B1-8 and F1-8) at various concentrations was calculated using Formula 1. The inhibitory concentration of 50% of α -glucosidase (IC_{50}) values was calculated from the graph of the percentage inhibition against extract concentrations.

Antioxidant activities

Determination of total phenolic contents

The Folin-Ciocalteu reagent was used to determine the total phenolic content according to the method described by Kähkönen et al.²⁴ Gallic acid was also used as standard compound. The total phenolic contents of all extracts (L1-8, B1-8 and F1-8) were expressed as mg gallic acid equivalents (GAE) per g of dry weight sample.

Determination of total flavonoid contents

The total flavonoid content was measured by using the aluminum nitrate assay (Chang et al.²⁵ 2002). Quercetin was used as the standard compound. The total flavonoid contents of all extracts (L1-8, B1-8 and F1-8) were expressed as mg quercetin equivalents (QE) per g of dry weight sample.

DPPH radical scavenging assay

The DPPH radical scavenging activities of all extracts (L1-8, B1-8 and F1-8) were examined using the method described by Blois compared with gallic acid and ascorbic acid as the reference compounds.²⁶ The absorbance of the sample (A_{sample})

was measured at 517 nm. An assay mixture without samples was used as a control (A_{control}). The inhibition percentage was calculated using Formula 2. The scavenging concentrations of 50% of DPPH (SC_{50}) values were calculated from the graph of the percentage inhibition against extract concentrations.

Formula 2.

$$\text{Scavenging effects (\%)} = \left[\frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \right] \times 100$$

PRAP assay

PRAP of all L1-8, B1-8 and F1-8 extracts were examined using phosphomolybdic acid.²⁷ The PRAP of extracts was expressed as mg QE per g of dry weight sample.

FRAP assay

FRAP of all L1-8, B1-8 and F1-8 extracts was examined using the method described by Oyaizu.²⁸ The ferric-reducing power of extracts was expressed as butylated hydroxyanisole equivalents (BHA-E) per g of dry weight sample.

Prevention of DNA oxidative damage

The protective effects of all L1-8, B1-8 and F1-8 extracts of *C. microphylla* against DNA oxidative damage induced by hydroxyl radical were monitored by the conversion of pBR322 to open circular form according to Yeung et al.²⁹ Total volume of reaction mixture (10 μ L) contained Tris-HCl buffer (pH 7.0), supercoiled plasmid pBR322 DNA (250 ng), 1 mM $FeSO_4$, 2% H_2O_2 and 125 μ g/mL of extracts. The mixtures were incubated at 37°C for 1 h. The reaction was stopped by adding 5 μ L of loading buffer (0.2% bromophenol blue, 4.5% sodium dodecyl sulfate, 0.2% xylene cyanol, 30% glycerol). The mixtures were then loaded on 0.8% agarose gel containing EB 1 mg/mL in TAE (Tris-acetate-EDTA). Electrophoresis was carried out at 100 V for 90 min. and the resulting image was visualized with BioRad Gel Doc XR system.

Statistical analysis

The experiments were performed in triplicate and the results are expressed as the mean \pm standard deviation. The statistical analysis was performed with SPSS 15.0 for Windows and Microsoft Excel for Windows 10. The differences between the extracts were evaluated using one-way analysis of variance followed by Duncan's multiple range tests. $P < 0.05$ was considered statistically significant.

RESULTS

Enzyme inhibition

AChE inhibition results of extracts of leaf, stem bark and fruit from *C. microphylla* are presented in Table 2. All of the extracts had low AChE inhibition values when compared with galanthamine with IC_{50} values of 7.34 ± 0.09 μ g/mL. However, among the tested extracts, B5 and B2 exhibited the highest AChE inhibitions with IC_{50} values of 204.02 ± 0.95 μ g/mL and 230.58 ± 3.18 μ g/mL, respectively. Some of the extracts (L8, B3, B7, F1, F3, F4, F6, F7 and F8) were inactive against AChE enzyme.

The results of the tyrosinase enzyme inhibitory effect of the extracts are given in Table 2. The lowest IC₅₀ values of the extracts indicate a higher inhibition effectiveness. All of the extracts from *C. microphylla* exhibited promising activity against tyrosinase compared with kojic acid. Methanol and ethanol extracts of stem bark of *C. microphylla* displayed remarkable tyrosinase inhibitory activities with IC₅₀ values of lower than 50 µg/mL. The B2 extract exhibited the highest tyrosinase inhibition with IC₅₀ values of 37.30±0.27 µg/mL (p<0.05), and B5 inhibited tyrosinase with IC₅₀ values of 37.41±0.17 µg/mL.

In this work, IC₅₀ values of α-glucosidase inhibition of *C. microphylla* extracts are presented in Table 2. A lower IC₅₀

Table 2. IC₅₀ (µg/mL) of acetylcholinesterase, tyrosinase and α-glucosidase inhibitory activities of leaf, bark, and fruit extracts from *C. microphylla*

Samples*	AChE	Tyrosinase	α-glucosidase
L1	349.14±1.34 ^a	59.19±0.14	90.35±1.32
L2	472.81±3.77	51.30±0.26	^b 15.78±0.14
L3	355.83±1.84	70.71±0.16	258.13±2.41
L4	932.83±2.31	49.31±0.13	191.36±1.92
L5	382.20±2.84	43.74±0.28	^b 29.92±0.26
L6	324.77±1.72	52.42±0.73	57.80±0.94
L7	513.35±2.37	145.80±0.51	167.94±1.36
L8	nd	142.42±1.42	270.64±2.42
B1	314.83±2.50	38.79±0.82	465.60±2.26
B2	230.58±3.18	^b 37.41±0.17	^b 38.25±0.51
B3	nd	41.52±0.35	164.95±1.32
B4	538.31±1.52	38.25±0.62	367.65±2.42
B5	204.02±0.95	^b 37.30±0.27	^b 39.63±0.62
B6	630.21±2.52	40.32±0.21	68.31±0.22
B7	nd	155.90±1.47	256.76±2.35
B8	298.41±1.36	144.47±0.31	^b 46.02±0.52
F1	nd	129.34±0.46	nd
F2	301.77±2.25	139.37±0.32	624.22±2.48
F3	nd	85.77±0.41	nd
F4	nd	62.11±0.58	465.12±3.42
F5	434.53±3.27	56.02±0.21	250.94±1.95
F6	nd	147.29±0.52	nd
F7	nd	165.75±0.47	nd
F8	nd	149.83±0.69	731.81±3.26
Galantamine	7.34±0.09	-	-
Kojic acid	-	24.01±0.02	-
Acarbose	-	-	31.92±0.08

*L: Leaf, B: Bark, F: Fruit, ^aValues expressed are means ± SD, ^b(p<0.05), nd: Not detected, AChE: Acetylcholinesterase

value indicates strong inhibitory activity. L2, L5, B2, B5 and B8 extracts exhibited significant (p<0.05) α-glucosidase inhibition as shown in Table 2. IC₅₀ values of L2, L5, B2, B5 and B8 extracts were found to 15.78±0.14, 29.92±0.26, 38.25±0.51, 39.63±0.62 and 46.02±0.52 µg/mL, respectively. On the other hand, F1, F3, F6, and F7 extracts had no α-glucosidase inhibition effects. All of the data of α-glucosidase inhibition indicated that L2, L5, B2, B5, and B8 extracts of *C. microphylla* could be effective hypoglycemic agents.

Antioxidant activities

The total phenolic contents of various extracts of *C. microphylla* leaves, stem barks, and fruits were determined from the gallic acid standard curve ($y=1.9251x + 0.3125$, $R^2=0.9967$) and expressed as mg GAE/g dry weight. The total phenolic contents of *C. microphylla* stem barks and leaves were in the range of 13.22±0.38 to 132.26±1.83 mg GAE/g dry weight and 30.93±0.64 to 85.26±1.60 mg GAE/g dry weight, whereas extracts of fruits exhibited 5.00±0.18 to 57.28±1.35 mg GAE/g dry weight as shown in Figure 1. B1 (123.11±2.38), B2 (132.26±1.83), B4 (111.84±2.19), B5 (120.40±2.89), and B6 (112.46±2.13) extracts contained more than 100 mg GAE/g dry weight. On the other hand, B7 and F8 extracts exhibited the lowest total phenolic contents (13.22±0.38, 5.00±0.18 and 14.89±0.73 mg GAE/g dry weight).

Total flavonoid contents of leaf, stem bark, and fruit extracts from *C. microphylla* were determined from the quercetin standard curve ($y=12.632x \pm 0.509$, $R^2=0.9981$) as shown in Figure 2. The total flavonoid contents expressed as mg QE/g dry weight found in our extracts ranged from 0.97±0.09 to 63.34±0.92 mg QE/g dry weight. Total flavonoid contents of leaf extract from *C. microphylla* appeared higher than other extracts. The highest total flavonoid content was found in the L1 (63.34±0.92 mg QE/g dry weight) extract, followed by the L2 (56.25±0.73 mg QE/g dry weight), L4 (52.89±0.47 mg QE/g dry weight), L5 (49.39±1.03 mg QE/g dry weight), and L6 (50.53±0.92 mg QE/g dry weight) extracts. Stem bark extracts of *C. microphylla* were in the range of 0.97±0.09 to 4.78±0.24 mg QE/g dry weight.

Among the tested extracts, B2 (9.89±0.09 µg/mL), B5 (10.47±0.29 µg/mL), B1 (11.94±0.07 µg/mL) and L2 (12.29±0.07 µg/mL)

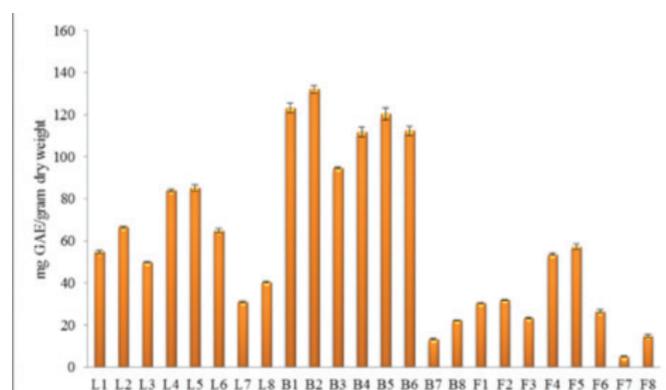


Figure 1. Total phenolic contents of the extracts

*L: Leaf, B: Bark, F: Fruit

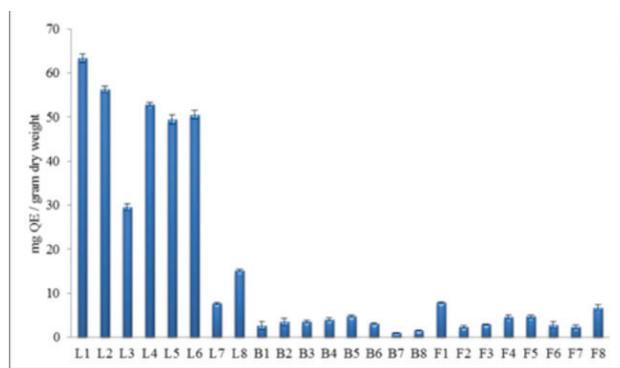


Figure 2. Total flavonoid contents of the extracts

*L: Leaf, B: Bark, F: Fruit

($p < 0.05$) extracts showed the highest scavenging activity in this assay as shown in Table 3. The IC_{50} values of ethanol, acidified ethanol, methanol, and acidified methanol extracts of leaf and stem bark of *C. microphylla* were found lower than 70 $\mu\text{g}/\text{mL}$. In the leaf, stem bark, and fruit extracts of *C. microphylla*, F7 extract showed the lowest DPPH radical scavenging activities. F5 extract exhibited the highest scavenging activities among the leaf extracts with $123.50 \pm 1.31 \mu\text{g}/\text{mL}$.

PRAP of leaf, stem bark, and fruit extracts from *C. microphylla* were determined from the quercetin standard curve ($y = 0.0066x \pm 0.5295$, $R^2 = 0.9986$) as shown in Table 3. B2, B5, and B4 extracts displayed the highest reducing activities with 368.37 ± 2.41 , 324.69 ± 3.69 and $247.75 \pm 2.73 \text{ mg QE}/\text{g dry weight}$,

Table 3. DPPH radical scavenging, phosphomolybdenum-reducing antioxidant power (PRAP) and ferric-reducing antioxidant power (FRAP) assay values of leaf, bark, and fruit extracts from *C. microphylla*

Sample*	DPPH radical scavenging [SC_{50} values of extracts ($\mu\text{g}/\text{mL}$)]	PRAP (mg QE/g dry weight)	FRAP (mg BHAE/g dry weight)
L1	23.42 ± 0.19^a	98.54 ± 1.35	165.58 ± 0.33
L2	12.29 ± 0.07	138.93 ± 1.42	214.87 ± 0.72
L3	20.77 ± 1.38	149.76 ± 0.42	227.00 ± 2.32
L4	17.94 ± 1.40	82.12 ± 0.57	222.04 ± 4.95
L5	15.79 ± 0.38	117.83 ± 2.52	197.07 ± 1.66
L6	42.83 ± 0.72	42.91 ± 0.93	205.58 ± 0.88
L7	149.12 ± 2.41	34.86 ± 0.36	123.69 ± 3.22
L8	91.40 ± 1.42	35.40 ± 0.39	140.29 ± 1.45
B1	11.94 ± 0.14	179.89 ± 1.63	191.77 ± 2.78
B2	9.89 ± 0.09	368.37 ± 2.41	240.62 ± 1.03
B3	34.04 ± 0.52	151.10 ± 1.58	216.20 ± 1.64
B4	18.84 ± 0.38	247.75 ± 2.73	224.30 ± 4.11
B5	10.47 ± 0.29	324.69 ± 3.69	232.26 ± 1.83
B6	65.52 ± 1.41	221.54 ± 2.51	199.70 ± 1.55
B7	140.92 ± 2.51	40.84 ± 0.93	59.20 ± 1.52
B8	60.06 ± 0.93	41.44 ± 0.83	57.24 ± 0.77
F1	177.11 ± 1.58	34.88 ± 0.25	60.28 ± 2.44
F2	164.63 ± 1.79	38.24 ± 0.49	62.84 ± 1.75
F3	206.16 ± 2.69	32.45 ± 0.41	53.11 ± 0.81
F4	131.53 ± 1.44	37.39 ± 0.18	76.29 ± 1.69
F5	123.50 ± 1.31	42.80 ± 0.15	87.28 ± 2.05
F6	219.97 ± 2.48	29.44 ± 0.17	35.39 ± 0.99
F7	669.21 ± 3.94	25.68 ± 0.82	25.00 ± 2.38
F8	600.70 ± 2.07	28.63 ± 0.41	34.99 ± 1.42
GA	68.14 ± 0.18	-	-
AA	54.01 ± 0.13	-	-

*L: Leaf, B: Bark, F: Fruit, ^aValues expressed are means \pm SD, ^b($p < 0.05$), BHAE: Butylated hydroxyanisole equivalents, QE: Quercetin equivalents, DPPH: 2,2-diphenyl-1-picrylhydrazyl

respectively; F7 extract indicated the lowest activity 25.68 ± 0.82 mg QE/g dry weight dry weight.

The results of the ability to reduce Fe^{3+} to Fe^{2+} are presented in Table 3. Stem bark and leaf extracts have a strong ferric reducing power. B2 and B5 extracts demonstrated the highest ferric reducing activity with 240.62 ± 1.03 mg BHA/g dry weight and 232.26 ± 1.83 mg BHA/g dry weight, respectively; F7 extract exhibited the lowest activity 25.00 ± 2.38 mg BHA/g dry weight.

Prevention of DNA oxidative damage

It is known that when circular plasmid DNA is subjected to electrophoresis, the fastest to migrate is the supercoiled Form I, the slowest moving is the open circular Form II, and the linear Form III runs in between the other two forms.³⁰ Prevention of DNA oxidative damage by *C. microphylla* is shown in Figure 3. The assay revealed that there was a formation of Form II and Form III because of hydroxyl radicals, as shown in Lane 2 on Figure 3.

However, with the addition of extracts, the conversion of supercoiled pBR322 DNA to open circular and linear forms decreased except with F8 extract at $125 \mu\text{g/mL}$. L4 and B4 extracts exhibited the highest preventative effect of DNA oxidative damage at $125 \mu\text{g/mL}$. The results proved that the prevention of DNA oxidative damage results were compatible with the radical scavenging assay.

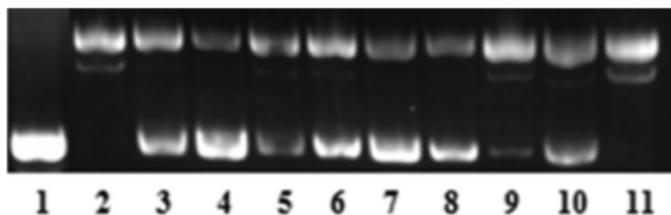


Figure 3. Protective effect of ethanol, methanol and water extracts of leaf, stem bark and fruit from *C. microphylla* in DNA oxidative damage assay. Lane 1: DNA control; Lane 2: DNA + 2% H_2O_2 + 1 mM $FeSO_4$; Lane 3: DNA + 2% H_2O_2 + 1 mM $FeSO_4$ + L1; Lane 4: DNA + 2% H_2O_2 + 1 mM $FeSO_4$ + L4; Lane 5: DNA + 2% H_2O_2 + 1 mM $FeSO_4$ + L7; Lane 6: DNA + 2% H_2O_2 + 1 mM $FeSO_4$ + B1; Lane 7: DNA + 2% H_2O_2 + 1 mM $FeSO_4$ + B4; Lane 8: DNA + 2% H_2O_2 + 1 mM $FeSO_4$ + B7; Lane 9: DNA + 2% H_2O_2 + 1 mM $FeSO_4$ + F1; Lane 10: DNA + 2% H_2O_2 + 1 mM $FeSO_4$ + F4; Lane 11: DNA + 2% H_2O_2 + 1 mM $FeSO_4$ + F7.

*L: Leaf, B: Bark, F: Fruit

DISCUSSION

Alzheimer's disease (AD) is one of the most frequent forms of dementia among older people.³¹ Although AChE inhibitors such as tacrine, donepezil, galantamine, and rivastigmine are important in the treatment for AD, they have adverse effects including gastrointestinal problems.^{32,33} Considering all the extracts, stem bark extracts, which had promising results at AChE inhibition, presented higher phenolic content than the other extracts (Figure 1). Recent studies have shown that antioxidants could scavenge oxygen radicals and could also attenuate inflammation pathways, and also pointed toward an association between AD and

inflammatory processes as well as antioxidant activity.³⁴ From this point of view, it is stated that the use of antioxidants could be considered in the treatment of AD.³⁵

Parkinson's disease (PD) is one of the neurodegenerative diseases caused by dopaminergic neuron deficiency in the brain.³⁶ Methanol and ethanol extracts from *C. microphylla* had higher inhibition activity than water extracts of *C. microphylla* due to total phenolic contents. There is a positive correlation between phenolic content and tyrosinase inhibition.³⁷ These results showed that, extracts of *C. microphylla*, especially B5 extract, had promising neuroprotective potential due to AChE and tyrosinase inhibition.

α -Glucosidase is a key enzyme in the hydrolysis of oligosaccharide and contributes to the formation of glucose.³⁸ It is important to find a new α -glucosidase inhibitor for DM, such as natural products with low toxicity and adverse effects.

Organic solvents such as methanol and ethanol are known to be efficient for the extraction of phenolics. Besides, water is a good choice because it is used to make infusions and decoctions in herbal medicine. Also, acidified extraction systems were shown to be more efficient, especially for the hydrolysis of bound phenolic compounds.^{39,40} Due to the fact that many solvents may extract different compounds from plant tissues, we wanted to compare the results. The hydrolysis process was done with acidification and aglycones were obtained with acidified extracts (L2, 5, 8; B2, 5, 8; F2, 5, 8) (Table 1).

When we compared the extracts that were prepared with the same solvents, total phenolic contents of the acidified ones were found to be higher than the non-acidified ones (Figure 1). The total phenolic content of L2 was found to be higher than L1, L5 was higher than L4, and L8 was higher than L7. The same results were also obtained with B and F series (Figure 1).

Similar to our findings, it was reported that methanol extract of *C. microphylla* leaves indicated scavenging activity to $92.82 \pm 0.79\%$ at $500 \mu\text{g/mL}$.⁴¹ According to Sharifi et al.⁴², IC_{50} values of methanol extract of *C. microphylla* were found as $13.01 \pm 0.2 \mu\text{g/mL}$.

The efficiency of an antioxidant extract was reported to be dependent on the pH of the solvents, as well as the solubility of antioxidant compounds by the solvents used for the extraction.⁴³ Besides, methanol, ethanol, and water, which are commonly used solvents for extraction, and acidified alcohols are also widely used for extraction to release aglycone by chemical hydrolysis under acidic conditions.⁴⁴ These results confirm that higher contents of total phenolic displayed higher DPPH free radical scavenging activities. All data showed that there was a relationship between the total phenolic and radical scavenging activities.

The results showed that methanol and ethanol extracts of leaf and bark from *C. microphylla* had more effective phosphomolybdenum-reducing power than its water extract. The B2 and B5 extracts with higher reducing power showed a positive correlation with phosphomolybdenum-reducing power assay.

Prevention of DNA oxidative damage was based on the ability of

extracts (L1-8, B1-8 and F1-8) from *C. microphylla* to protect the supercoiled pBR322 DNA against damage caused by hydroxyl radicals ($\cdot\text{OH}$). The antioxidant activity of 50% aqueous methanolic extract of whole plant of *C. microphylla* was studied before with an *in vitro* study and found to have moderate antioxidant activity.⁴⁵ However, there are no previous works on the AChE, tyrosinase, α -glucosidase inhibitory effects and oxidative DNA damage protective effects of various extracts of *C. microphylla*. In this context, it was aimed to compare the extractability of compounds that serve a function in the activity by various solvents.

CONCLUSION

This study presented the potential AChE, tyrosinase, α -glucosidase inhibitory effects, total phenolic, total flavonoid contents, the antioxidant effects, and prevention of oxidative DNA damage of leaf, stem bark and fruit of various extracts (L1-8, B1-8 and F1-8) from *C. microphylla*. Concurrently, the correlation between the antioxidant activity and the DNA damage protective effects of the extracts (L1-8, B1-8 and F1-8) was described. Our results can be evaluated as a preliminary work for the use of *C. microphylla* extracts in herbal products.

Conflict of Interest: No conflict of interest was declared by the authors.

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