The in vitro protection by *Crataegus microphylla* extracts against oxidative damage and enzyme inhibition effects

Short title: Biological activities of *Crataegus microphylla*

ABSTRACT

Objectives: *Crataegus* species have been used as food and also in folk medicine for the treatment of various diseases. The present study aims to make investigations on biological 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 seperated 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 biological effects such as prevention of oxidative DNA damage, acetylcholinesterase, tyrosinase, α-glucosidase inhibition and antioxidant effects with 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, phosphomolybdenum-reducing antioxidant power (PRAP), and ferric-reducing antioxidant power (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 other extracts with IC_{50} values of 204.02 ± 0.95 µg/mL and 37.30 ± 0.27 µg/mL, respectively. Acidified ethanol extract of leaves, was the most efficient extract against *α*-glucosidase, giving IC_{50}; 15.78 ± 0.14 µg/mL. IC_{50} value of the acidified ethanol extract for DPPH was 9.89 ± 0.09 µg/mL. Methanol extracts of leaves and stem barks at the dose of 125 µg/mL, exhibited significant protecting activity against DNA strand scission by hydroxyl radicals (·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.

Keywords: DPPH, FRAP, hawthorn, PRAP, Rosaceae.

Crataegus microphylla ekstrelerinin oksidatif hasara karşı in vitro koruma enzim inhibisyonu etkileri

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 HC1 , 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, fosfomolibdenyum indirgeyici antioksidan güç (PRAP) ve ferrik indirgeyici antioksidan güç (FRAP) gibi çeşitli biyolojik aktiviteleri farklı konsantrasyonlarda araştırılmıştır.

Bulgular: Gövde kabuklarından asitlendirilmiş metanol ekstraktı sırasıyla 204.02 ± 0.95 µg/mL ve 37.30 ± 0.27 µg/mL IC_{50} değerleri ile diğer esktreler arasında en yüksek asetilkolinesteraz ve tirozinaz inhibisyonu göstermiştir. Yaprakların asitlendirilmiş etanol ekstresi, α-glukosidaz enzimine karşı 15.78 ± 0.14 µg/mL ile en düşük IC_{50} değerini göstermiştir. DPPH için asitleştirilmiş etanol ekstraktının IC_{50} değeri 9.89 ± 0.09 ug/mL bulunmuştur. 125 µg/mL dozunda yaprakların ve gövde kabuklarının metanol ekstreleri, süpersarmal pBR322 DNA üzerinde hidroksil iyonu ($^{\cdot}OH$) 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.

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 *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). Also products that include the extracts of some *Crataegus* species consumed as natural health products in Europe, Asia, and North America (7,8). Epidemiological studies and associated meta-analyses showed that long term consumption of plant polyphenols in diet, cause protection against development of cancers, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases (9-13).

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

By the results of many pharmacological studies that performed with extracts and isolated constituents of *Crataegus* species; flavonoids and proantocyanidins were found to be responsible from the cardiovascular preventive 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). Also hyperoside was found to be the major compound in leaves and flowers of *C. microphylla* (17).

Oxidative stress has been involved in several neurodegenerative disease and degenerative disorders such as cancer, arteriosclerosis and diabetes etc. (19). As the accepted consent, phenolic content determines the antioxidative properties of plant species and polyphenols play 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. Especially 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 form with various extracts from *C. microphylla*.

In this study, prevention of oxidative DNA damage, acetylcholinesterase, 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 extracted with ethanol, methanol and water were investigated. The biological 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 and fruit of *C. microphylla* were collected from Kale, Gümüşhane-Turkey, in September 2015. The voucher specimen was deposited at the Hacettepe University Faculty of Pharmacy Herbarium (Voucher No: HUEF 15021).

Dried leaf (L), stem bark (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 carried out in a shaker for 4 h x 3 times, for each sample. Extracts were filtered and evaporated under reduced pressure using rotary evaporator. Crude extracts were kept in a refrigerator at + 4°C until used. All of the extracts were tested within the all assays.

Enzyme Inhibitions

Acetylcholinesterase (AChE) Inhibition

Acetylcholinesterase (AChE) inhibition was examined using the method described by Ellman et al. and Ingkaninan et al. (20,21). Galantamine was used as the positive control. All extracts (**L1-8**, **B1-8** and **F1-8)** at various concentrations were separately added in a 96-well microplate and incubated for 15 min at 25°C. The absorbance was measured at 412 nm using a 96-well microplate reader. Inhibition of AChE was calculated by using the formula 1. $A_{control}$ 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 concentration of 50% of AChE (IC_{50}) values were calculated from the graph of the percentage inhibition against extract concentrations.

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

Tyrosinase Inhibition

Tyrosinase inhibition was examined using the method described by Masuda (22). Kojic acid was used as the positive control. The tyrosinase inhibition percentage of all extracts (**L1-8, B1-8** and **F1-8)** (20 µL) at various concentrations, was calculated using the 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 the 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 utilizing the aluminum nitrate assay (Chang et al. 2002) (25). Quercetin was also 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 to gallic acid and ascorbic

acid as the reference compounds (26). The absorbance of the sample (A_{sample}) was measured at 517 nm. Assay mixture without samples was used as a control ($A_{control}$). The inhibition percentage was calculated using the formula 2. The scavenging concentration of 50% of DPPH (SC_{50}) values were calculated from the graph of the percentage inhibition against extract concentrations.

Formula 2. $$Scavenging\ effects\ (\%) = \left[\frac{(A_{control} - A_{sample})}{A_{control}}\right] \times 100$$

Phosphomolybdenum-Reducing Antioxidant Power (PRAP) Assay

PRAP of all **L1-8, B1-8** and **F1-8** extracts were examined using phosphomolybdic acid (27). The phosphomolybdenum-reducing antioxidant power of extracts was expressed as mg quercetin equivalents (QE) per g of dry weight sample.

Ferric-Reducing Antioxidant Power (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 hdroxyanisole equivalents (BHAE) 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 which was 10 µL, contained Tris-HCl buffer (pH 7.0), supercoiled plasmid pBR322 DNA (250 ng), 1 mM $FeSO_4$, 2% H_2O_2 and 125 µg/mL of extracts. The mixtures were incubated at 37 ºC for 1 h. The reaction was stopped by adding 5 µL 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 resulting image was visualized with BioRad Gel Doc XR system.

Statistical Analysis

The experiments were carried out in triplicate and results were expressed as the mean ± standard deviation (SD). The statistical analysis was performed with SPSS 15.0 for Windows and Microsoft Excel for Windows 10. The differences among the extracts were evaluated by one-way analysis of variance (ANOVA) 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* were presented in Table 2. All of the extracts had low AChE inhibition values when compared to galanthamine with IC_{50} values of 7.34 ± 0.09 µg/mL. However, among the tested extracts; **B5** and **B2** exhibited the highest AChE inhibitions with IC_{50} values of 204.02 ± 0.95 µg/mL and 230.58 ± 3.18 µg/mL, respectively. Some of extracts (**L8**, **B3**, **B7**, **F1**, **F3**, **F4**, **F6**, **F7** and **F8**) were inactive against AChE enzyme.

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

In this work, IC_{50} values of *α*-glucosidase inhibition of *C. microphylla* extracts were presented in Table 2. A lower IC_{50} 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. IC_{50} 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* can be effective hypoglcemic agents.

Antioxidant Activities

The total phenolic contents of various extracts of *C. microphylla* leaves, stem barks and fruits were determined from 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 – 132.26 ± 1.83 mg GAE/g dry weight and 30.93 ± 0.64 – 85.26 ± 1.60 mg GAE/g dry weight, whereas extracts of fruits exhibited 5.00 ± 0.18 – 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 quercetin standard curve (y=12.632x ± 0.509, R^2=0.9981) as shown in Figure 2. The total flavonoid contents expressed as mg quercetin equivalents/g dry weight found in our extracts varied 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 – 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) ($p < 0.05$) extracts showed the highest scavenging activity in this assay as shown in Table 3. 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 µg/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 ± 1.31 µg/mL.

PRAP of leaf, stem bark, and fruit extracts from *C. microphylla* were determined from quercetin standard curve (y=0.0066x ± 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 ± 2.73 mg QE/g dry weight, respectively. On the other hand, **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+} were 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 BHAE/g dry weight and 232.26 ± 1.83 mg BHAE/g dry weight, respectively. On the other hand, **F7** extract exhibited the lowest activity 25.00 ± 2.38 mg BHAE/g dry weight.

Prevention of DNA Oxidative Damage

It has been known that when circular plasmid DNA was subjected to electrophoresis, the fastest migrating was the supercoiled Form I, the slowest moving was 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* was shown on 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 4. However, with the addition of extracts, conversion of supercoiled pBR322 DNA to open circular and lineer form decreased except **F8** extract at 125 µg/mL. **L4** and **B4** extracts exhibited the highest preventive effect of DNA oxidative damage at 125 µg/mL. The results proved that prevention of DNA oxidative damage results were compatible with radical scavenging assay.

DISCUSSION

Alzheimer disease (AD) is one of the most frequent forms of dementia among old age people (31). Although, acetylcholinesterase (AChE) inhibitors such as tacrine, donepezil, galantamine and rivestigmine are important in the treatment for AD, they have side effects containing gastrointestinal problems (32,33). Considering all the extracts, stem bark extracts which had promising results at AChE inhibition, presented higher phenolic content among the other extracts (Figure 1). Recent studies have showed that antioxidants can scavenge oxygen radicals and can also attenuate inflammation pathways, and also have pointed the 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 disease (PD) is the one of neurodegenerative diseases due to dopaminergic neurons 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 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 acetylcholinesterase and tyrosinase inhibition.

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

Organic solvents such as methanol and ethanol are known to be efficient for the extraction of phenolics. Besides, water is a good choice as it is used to make infusions and decoctions in herbal medicine. Also the acidified extraction systems was 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 the plant tissues, we wanted to compare the results. Hydrolysation process was done with acidification and aglycones were obtained with acidified extracts (L2,5,8; B2,5,8; F2,5,8) (Table1). 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 (Figure1). The total phenolic content of L2 was found to be higher than L1, L5 was higher than L4 and L8 was higher than L7. Same results were also obtained with B and F series (Figure 1).

In similar with our findings, it was reported that methanol extract of *C. microphylla* leaves indicated scavenging activity to 92.82 ± 0.79% at 500 µg/mL (41). According to Sharifi et al, IC_{50} values of methanol extract of *C. microphylla* were found as 13.01 ± 0.2 µg/mL (42).

The efficiency of an antioxidant extract was reported to be dependent on the pH of the solvents and as well as the solubility of antioxidant compounds by the solvents used for the extraction (43). Besides, methanol, ethanol and water which were commonly used solvents for the extraction, acidified alcohols are also widely used for extraction to release the 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 of data showed that there is 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* have more effective phosphomolybdenum-reducing power than water extracts of it. The **B2** and **B5** extracts with higher reducing power showed 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 (·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). But, there were no previous works on the acetylcholinesterase, 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 the compounds that serve a function in the activity by various solvents.

CONCLUSION

This study presented the potential acetylcholinesterase, 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 preliminary work for the usage of *C. microphylla* extracts in herbal products.

REFERENCES

1. Dönmez AA. The Genus *Crataegus* L. (Rosaceae) with special reference to hybridisation and biodiversity in Turkey. Turk J Bot 28, 29-37, 2004.
2. Dönmez AA. A new species of *Crataegus* (Rosaceae) from Turkey. Bot J Linn Soc 148, 245–249, 2005.
3. Chang Q, Zhong Z, Harrison F, Chow MSS, Hawthorn. J Clin Pharmacol 42, 605-612, 2002.
4. Browicz K. Crataegus L. In: PH Davis (ed). Flora of Turkey and the East Aegean Islands, Edinburgh University Press: Edinburgh, 4, 133-147, 1972.
5. Kumar D, Arya V, Bhat ZA, Khan NA, Prasad DN, The genus *Crataegus*: chemical and pharmacological perspectives. Rev Bras Farmacogn 22, 1187-1200, 2012.
6. Rastogi S, Pandey MM, Rawat AK. Traditional herbs: a remedy for cardiovascular disorders. Phytomed 23, 1082-1089, 2015.
7. Edwards JE, Brown PN, Talent N, Dickinson TA, Shipley PR. A review of the chemistry of the genus *Crataegus*. Phytochemistry 79, 5-26, 2012.
8. Assessment report on *Crataegus* spp., folium cum flöre, European Medicines Agency Committee on Herbal Medicinal Products (HMPC), EMA/HMPC/159076/2014, accessed on 13 October 2014.
9. Pandey KB, Rizvi SI. Plant polyphenols as dietary antioxidants in human health and disease. Oxi. Med. Cell. Longevity 2 (2009) 270–278.
10. Abidi E, Habib J, Yassine A, Chahine N, Mahjoub T, Ellak A. Effects of methanol extracts from roots, leaves, and fruits of the Lebanese strawberry tree on cardiac function together with their antioxidant activity. Pharm Biol 54, 1035-1041, 2016.
11. Graf BA, Milbury PE, Blumberg JB. Flavonols, flavones, flavanones, and human health: epidemiological evidence. J Med Food 8(3), 281-290, 2005.

12. Arts IC, Hollman PC. Polyphenols and disease risk in epidemiologic studies. Am J Clin nutr 81, 317S-325S, 2005.
13. Palma-Duran SA, Vlassopoulos A, Lean M, Govan L, Combet E. Nutritional intervention and impact of polyphenol on glycohemoglobin (HbA1c) in non-diabetic and type 2 diabetic subjects: Systematic review and nmeta-analysis. Crit Rev Food Sci Nutr 57, 975-986, 2017.
14. Hosseinimehr SJ, Azadbakht M, Tanha M, Mahmodzadeh A, Mohammadifar S. Protective effect of hawthorn extract against genotoxicity induced by methyl methanesulfonate in human lymphocytes. Toxicol and Health 27, 363-369, 2011.
15. Strain HH. Sources of d-sorbitol. J Am Chem Soc 59, 2264-2266, 1937.
16. Meriçli AH, Melikoğlu G. Investigations on Turkish *Crataegus* species. Acta Pharm Turcica 44, 169-173, 2002.
17. Melikoğlu G, Bitiş L, Meriçli AH. Flavonoids of *Crataegus microphylla*. Nat Prod Res 18, 211-213, 2004.
18. Tajali AA, Khazaeipool M. Effects of height and organs on flavonoids of *Crataegus microphylla* C. Koch in Iran. Int J Biosci 2, 54-58, 2012.
19. Moyo MS, Amoo O, Ncube B, Ndhlala AR, Finnie JF, Van Staden J. Phytochemical and antioxidant properties of unconventional leafy vegetables consumed in Southern Africa. S Afr J Bot 84, 65-71, 2013.
20. Ellman G, Courtney K, Andres V, Featherstone RM. A new colorimetric determination acetylcholinesterase activity. Biochem Pharmacol 7, 88–95, 1961.
21. Ingkaninan K, Best D, Heijden VD, Hofte AJP, Karabatak B, Irth H, Tjaden UR, Greef VD, Verpoorte R. High-performance liquid chromatography with on-line coupled UV, mass spectrometric and biochemical detection for identification of acetylcholinesterase inhibitors from natural products. J Chrom 872, 61–73, 2000.
22. Masuda T, Yamashita D, Takeda Y, Yonemori S. Screening for tyrosinase inhibitors among extracts of seashore plants and identification of potent inhibitors from *Garcinia subelliptica*. Biosci Biotech Bioch 69, 197-201, 2005.
23. Da Silva Pinto M, Kwon Y, Apostolidis E, Lajolo F, Genovese MI, Shetty K. Functionality of bioactive compounds in brazilian strawberry (*Fragaria* x A*nanassa* Duch.) cultivars: evaluation of hyperglycemia and hypertension potential using *in vitro* models. J Agr Food Chem 56, 4386–4392, 2008.

24. Kähkönen MP, Hopia AI, Vuorela HJ, Rauha JP, Pihlaja K, Kujala TS, Heinonen M. Antioxidant Activity of Plant Extracts Containing Phenolic Compounds. J Agr Food Chem 47, 3954-3962, 1999.
25. Chang CC, Yang MH, Wen HM, Chern JC. Estimation of total flavonoid content by omplementary colorimetric methods. J Food Drug Anal 10, 178-182, 2002.
26. Blois MS. Antioxidant determinations by the use of a stable free radical, Nature, 181, 1199-1200, 1958.
27. Falcioni G, Fedeli D, Diano L, Calzuola I, Mancinelli L, Marsili V, Gianfranceshi G. Antioxidant activity of wheat sprouts extract *in vitro*: inhibition of DNA oxidative damage. J Food Sci 67, 2918-2922, 2002.
28. Oyaizu M. Studies on products of browning reactions-antioxidative activities of browning reaction prepared from glucosamine. Jpn J Nut 44, 307-315, 1986.
29. Yeung SY, Lan WH, Huang CS, Lin CP, Chan CP, Chang MC, Jeng JH. Scavenging property of three cresol isomers against H_2O_2, hypochlorite, superoxide and hydroxyl radicals. Food Chem Toxicol 40, 1403-1413, 2002.
30. Özel A, Barut B, Demirbaş Ü, Biyiklioglu Z. Investigation of DNA binding, DNA photocleavage, topoisomerase I inhibition and antioxidant activities of water soluble titanium(IV). J Photochem Photobiol B 57, 32-38, 2016.
31. Lewis WG, Green LG, Grynszpan F, Radic Z, Carlier PR, Taylor P, Finn MG, Sharpless KB. Click chemistry in situ: acetylcholinesterase as a reaction vessel for the selective assembly of a femtomolar inhibitor from an array of building blocks. Angew Chem Int Ed 41, 1053-1057, 2002.
32. Tian T, Weng L, Wang S, Weng X, Zhang L, Zhou X. Cationic tetrapyrrolic macromolecules as new acetylcholinesterase inhibitors. J Porphyr Phthalocyanines 13, 893-902, 2009.
33. Zengin G, Nithiyanantham S, Locatelli M, Ceylan R, Uysal S, Aktumsek A, Kalai Selvi P, Maskovic P. Screening of *in vitro* antioxidant and enzyme inhibitory activities of different extracts from two uninvestigated wild plants. Eur J Integr Med 8, 286-292, 2016.
34. Ferreira A, Proença C, Serralheiro MLM, Araujo MEM. The *in vitro* screening for acetylcholinesterase inhibition and antioxidant activity of medicinal plants from Portugal. J Ethnopharmacol 108, 31-37, 2006.
35. Gibson GE, Huang HM. Oxidative stress in Alzheimer's disease. Neurobiol Aging 26, 575–578, 2005.

36. Bao K, Dai Y, Zhu Z, Tu FJ, Zhang W, Yao X. Design synthesis biphenyl derivatives mushroom tyrosinase inhibitors. Bioorg Med Chem 18, 6708-6714, 2010.

37. Choi HK, Lim YS, Kim YS, Park YS, Lee CH, Hwang KW, Kwon DY. Free-radical-scavenging and tyrosinase-inhibition activities of Cheonggukjang samples fermented for various time. Food Chem 106, 564-568, 2008.

38. Majouli K, Hlila MB, Hamdi A, Flamini G, Jannet HB, Kenani A. Antioxidant activity and α-glucosidase inhibition by essential oils from *Hertia cheirifolia* (L.). Ind Crop Prod 82, 23-28, 2016.

39. Acosta-Estrada BA., Gutierrez-Uribe JA, Serna-Saldivar SO. Bound phenolics in foods, a review. Food Chem 152, 46-55, 2014.

40. Mushtaq M, Sultana B, Anwar F, Batool S. Antimutagenic and antioxidant potential of aqueous and acidified methanol extracts from citrus limonum fruit residues. J Chil Chem Soc 60, 2979-2983, 2015.

41. Karagöz A, Artun FT, Özcan G, Melikoğlu G, Anıl S, Kültür Ş, Sütlüpınar N. *In vitro* evaluation of antioxidant activity of some plant methanol extracts. Biotechnol Biotec Eq 29, 1184-1189, 2015.

42. Sharifi N, Souri E, Ziai SA, Amin G, Amanlou M. Discovery of new angiotensin converting enzyme (ACE) inhibitors from medicinal plants to treat hypertension using on *in vitro* assay. DARU Journal 21, 1-8, 2013.

43. Kutlu T, Durmaz G, Ateş B, Yilmaz I, Çetin MŞ. Antioxidant properties of different extracts of black mulberry (*Morus nigra* L.). Turk J Biol 35, 103-110, 2011.

44. Nollet LML, Toldra F. Handbook of Analysis of Active Compounds in Functional Foods, CRC Press: Boca Raton, 2012.

45. Serteser A, Kargıoğlu M, Gök V, Bağcı Y, Özcan MM, Arslan D. Determination of antioxidant effects of some plant species wild growing in Turkey. Int J Food Sci Nutr 59, 643-651, 2008.

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_2O (1:1)	**L3**	23.2
Leaf in MeOH	**L4**	20.6
Leaf in MeOH, pH 2.5	**L5**	17.6
Leaf in MeOH:H_2O (1:1)	**L6**	24.6
Leaf in H_2O	**L7**	10.2
Leaf in H_2O, 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_2O (1:1)	**B3**	9.1
Bark in MeOH	**B4**	10.0
Bark in MeOH, pH 2.5	**B5**	8.3
Bark in MeOH:H_2O (1:1)	**B6**	9.6
Bark in H_2O	**B7**	7.3
Bark in H_2O, 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_2O (1:1)	**F3**	6.3
Fruit in MeOH	**F4**	10.7
Fruit in MeOH, pH 2.5	**F5**	9.8
Fruit in MeOH:H_2O (1:1)	**F6**	5.7
Fruit in H_2O	**F7**	8.6
Fruit in H_2O, pH 2.5	**F8**	9.5

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

Table 2. IC_{50} (µg/mL) of aceytlcholinesterase, 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
[a]Values expressed are means ± SD
[b]($p < 0.05$)
nd: not detected

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 (µg/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	[b]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	[b]11.94 ± 0.14	179.89 ± 1.63	191.77 ± 2.78
B2	[b]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	[b]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
[a]Values expressed are means ± SD
[b]($p < 0.05$)

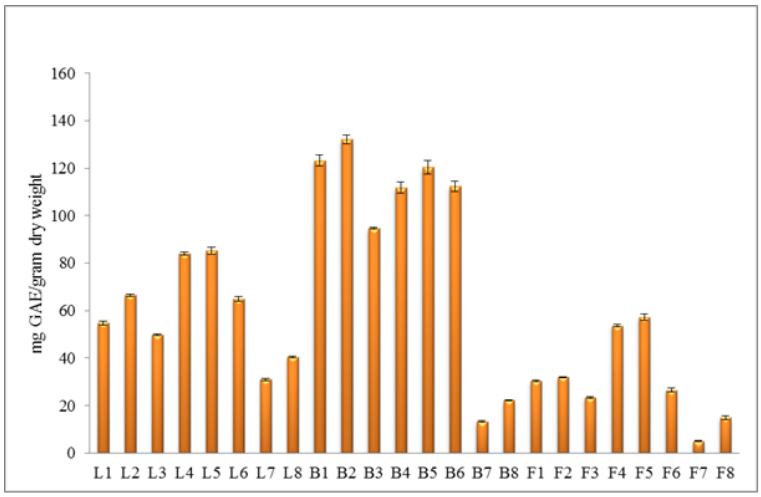

Figure 1. Total phenolic contents of the extracts

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

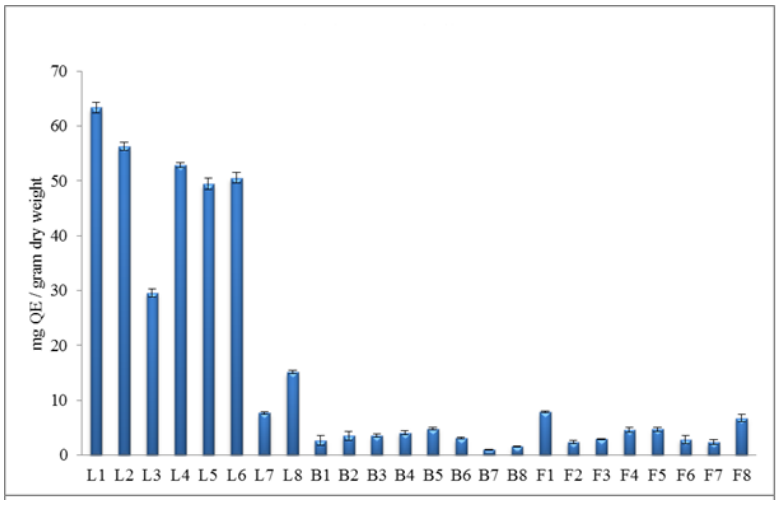

Figure 2. Total flavonoid contents of the extracts

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

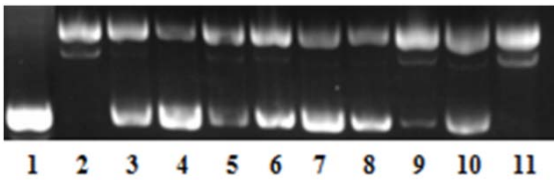

Figure 3. Protective effect of ethanol, methanol and water extracts of leaf, stem bark and fruit from *C. microphylla* in DNA oxidative damage assay. Lane 1: DNA control; Lane 2: DNA + 2% H_2O_2 + 1 mM $FeSO_4$; Lane 3: DNA + 2% H_2O_2 + 1 mM $FeSO_4$ + **L1**; Lane 4: DNA + 2% H_2O_2 + 1 mM $FeSO_4$ + **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)