

# Evaluation of phenolic profiles and antioxidant activities of Turkish medicinal plants: *Tilia argentea*, *Crataegi folium* leaves and *Polygonum bistorta* roots

S. Demiray, M.E. Pintado, P.M.L. Castro

**Abstract**— There is a growing interest in the food industry and in preventive health care for the development and evaluation of natural antioxidants from medicinal plant materials. In the present work, extracts of three medicinal plants (*Tilia argentea*, *Crataegi folium* leaves and *Polygonum bistorta* roots) used in Turkish phytotherapy were screened for their phenolic profiles and antioxidant properties. Crude extracts were obtained from different parts of plants, by solid-liquid extraction with pure water, 70% acetone and 70% methanol aqueous solvents. The antioxidant activity of the extracts was determined by ABTS<sup>+</sup> radical cation scavenging activity. The Folin Ciocalteu procedure was used to assess the total phenolic concentrations of the extracts as gallic acid equivalents. A modified liquid chromatography-electro spray ionization-mass spectrometry (LC-ESI-MS) was used to obtain chromatographic profiles of the phenolic compounds in the medicinal plants. The predominant phenolic compounds detected in different extracts of the plants were catechin, protocatechuic and chlorogenic acids. The highest phenolic contents were obtained by using 70% acetone as aqueous solvent, whereas the lowest phenolic contents were obtained by water extraction due to Folin Ciocalteu results. The results indicate that acetone extracts of *Tilia argentea* had the highest antioxidant capacity as free ABTS radical scavengers. The lowest phenolic contents and antioxidant capacities were obtained from *Polygonum bistorta* root extracts.

**Keywords**— Medicinal plants, antioxidant activity, total phenolics, LC-ESI-MS.

## I. INTRODUCTION

PLANTS have been an important source of medicine for thousands of years. Even today, the World Health

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Organization estimates that up to 80 per cent of people still rely mainly on traditional remedies such as herbs for their medicines [1]. Medicinal plants constitute one of the main sources of new pharmaceuticals and healthcare products. A whole range of plant-derived dietary supplements, phytochemicals and pro-vitamins that assist in maintaining good health and combating disease are now being described as functional ingredients and nutraceuticals. The role of medicinal plants in disease prevention or control has been attributed to antioxidant properties of their constituents, usually associated to a wide range of amphipathic molecules, broadly termed polyphenolic compounds [2].

The number of reports on the isolation of natural antioxidants, mainly of plant origin, has increased immensely during the last decade [3]. Polyphenolic compounds are commonly found in both edible and inedible plants, they have multiple applications in food, cosmetic and pharmaceutical industries [4]. The antioxidant capacity of phenolic compounds is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers or metal chelators. In addition to their roles as antioxidants, these compounds exhibit a wide spectrum of medicinal properties, such as anti-allergic, anti-inflammatory, anti-microbial, anti-thrombotic, cardio-protective and vasodilatory effects [5].

Medicinal plants are widely used in everyday life as part of folk medicinal remedies in Turkey. Turkey's flora is remarkable for its diversity and it is a rich source of medicinal plants. The aim of the present study was to assess the antioxidant activities of the medicinal plants; *Crataegi folium* leaves, *Polygonum bistorta* roots and *Tilia argentea* using the ABTS assay, to estimate the phenolic content using the Folin-Ciocalteu method and to determine the phenolic profile by LC-ESI-MS.

## II. METHODS AND MATERIALS

### A. Medicinal Plants

In the present study, three different, commercial, pre-

packaged, dry medicinal plants (*Tilia argentea*, *Crataegi folium* (Hawthorn) leaves, *Polygonum (Radix) bistorta* roots) used in Turkish phytotherapy were purchased from a local market in Ankara, Turkey. Selected plants are usually used in practice for the treatment of different disorders and available with no need for medical prescription, often as herbal infusion for everyday use.

#### B. Reagents and Standards

Acetone was analytical reagent grade and purchased from Merck (Darmstadt, Germany). Methanol was HPLC grade and purchased from Romil (Cambridge, UK). Folin-Ciocalteu reagent and ascorbic acid were from Merck (Darmstadt, Germany). 2,2-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in the crystallized diammonium salt form and gallic acid were obtained from Sigma-Aldrich Quimica (Alcobendas, Spain). Double distilled water (Millipore Co.) was used throughout.

HPLC grade phenolic standards (naringenin, catechin, quercetin, protocatechuic, caffeic, gallic, chlorogenic, ferulic and p-coumaric acids) were purchased from Merck (Darmstadt, Germany).

#### C. Extraction of Phenolic Compounds

Different solvent systems with increased polarities were used to evaluate the effectiveness of the solvent type on the extraction of phenolic compounds: 70% aqueous solutions of acetone, methanol and 100% pure water. The extracts were prepared by using 0.5 g of dry-ground sample and 20 mL of each solvent. The mixtures were shaken in a c24k refrigerated incubator shaker (NJ, USA) at room temperature for 60 min. Then, the mixtures were centrifuged in a Universal 320r Hettich Zenrifugen (Tuttlingen, Germany) at 9000 rpm for 5 min. at 4°C. The supernatant was recovered and used for the determination of total phenolic contents, antioxidant activities and LC-ESI/MS analysis. The supernatants were filtered through a 45µm filter prior to analysis.

All the extractions were performed in duplicate and the supernatants were kept at -20°C until further analysis.

#### D. Phenolic Content Determination

The total phenolic content of extracts was determined by using the Folin-Ciocalteu reagent according to a modification of the Parejo's method [6]. Solvent extract (0.5 ml), 0.5 ml of Folin-Ciocalteu reagent, 10 ml of 75g L-1 sodium carbonate and deionized water were added to a final volume of 25 ml. After 1 h, the absorbance of the sample was measured at 725 nm against a blank by a UV 1203 Shimadzu spectrophotometer (Tokyo, Japan). Gallic acid was used as the standard for preparing the calibration curve. All determinations were performed in triplicate.

#### E. Evaluation of Antioxidant Activity

The free radical-scavenging activity was determined by the ABTS radical cation decolorization assay described by Re et al. [7]. ABTS was dissolved in water to a 7 µM concentration. ABTS radical cation (ABTS.+ ) was produced by reacting

ABTS stock solution with 2.45 µM potassium persulfate (final concentration), mixture kept in the dark at room temperature for 12–16 h before use. The radical was stable in this form for more than two days when stored in the dark at room temperature. For the study of infusion, the samples containing the ABTS.+ solution were diluted with redistilled water to an absorbance of 0.700 (±0.02) at 734 nm and equilibrated at ambient temperature. A reagent blank reading was taken (A0). 2.0 ml of diluted ABTS.+ solution ( $A_{734nm} = 0.700 \pm 0.02$ ) were added to 20 µl of the extracts, and the absorbance was read 6 minutes after initial mixing (At). The results were corrected for dilution and were expressed in mg of ascorbic acid equivalents per g dry weight of plant sample. All determinations were performed in triplicate.

#### F. LC-ESI/MS Analysis

The chromatographic system was consisted of a Prostar 210 LC pump (Varian, CA, USA) coupled with a Varian 1200 triple quadrupole mass spectrometer (Varian, CA, USA) with electrospray ionization in positive and negative modes. A 5 µm C18 column (4.6mmx100mm, Merck) was used for the separation at a flow rate of 0.4 mL/min. For the analysis, a LC/MS/MS method that has been developed by Sun et al. [8] was modified. The separation was performed by gradient elution (eluent A, water with 0.1% formic acid; eluent B, 100% methanol) in 33 minutes. For MS/MS fragmentation, Argon atoms were used (pressure 1.20 mtorr; collision energy of 15 V). Data were acquired by Varian LC-MS 1200L Workstation.

#### G. Identification of Phenolic Compounds

LC and MS-MS library containing 23 phenolic compounds was established by ESI-LC/MS under negative ion mode. Structural identification of individual compounds in extracts was performed by comparison of the retention times and mass spectra of the pure phenolic standards [8, 9]. ESI-MS with different fragmentor voltage in the range of 10V to 120V has been tried.

#### H. Statistical Analysis

Results for total phenolic content and antioxidant activities were reported as means ± SD. Significant differences for multiple comparisons were determined by one-way analysis of variance (ANOVA) followed by Tukey's test with  $\alpha=0.01$  by SPSS statistical package (ver.12.0).

### III. RESULTS

Three medicinal plant species selected based on ethno botanical information compiled from Turkish folk medicine, have been evaluated for their phenolic profiles and antioxidant activities. In this study, the effect of various solvents on the extraction efficiency of phenolics was investigated. Differences between extraction solvents were monitored via total phenolic content, total antioxidant activity and composition of phenolics determined by LC-ESI-MS.

A simple and gradient elution-based ESI-LC/MS method was modified for the analysis and quantification of phenolic compounds in various extracts obtained from the medicinal plants. For the development of an effective mobile phase, various solvent systems, including combinations of methanol and water with different buffers were tried. A solvent system consisting of 0.01% formic acid in water and methanol proved successful, allowing the separation of phenolic compounds present in the plant extracts with good resolution. Results of identification as well as retention times and fragments are shown in Table 1.

TABLE I  
LC-ESI/MS SPECTRAL INFORMATION OF IDENTIFIED PEAKS

COMPOUND NAME	MW	[M-H]	RT (MIN)
<b>PHENOLIC ACIDS</b>			
<i>Hydroxybenzoic acids</i>			
Protocatechuic acid	154	153(109)	8.48
<i>Hydroxycinnamic acids</i>			
p-coumaric acid	164	163(119)	20.86
Ferulic acid	194	193(134)	22.75
Chlorogenic acid	354	353(191)	15.82
<b>FLAVONOIDS</b>			
<i>Flavan-3-ols</i>			
(+)-Catechin	290	289(245)	13.49
<i>Flavonol and glycosides</i>			
Quercetin	302	301(151)	27.71
<i>Flavanone and glycosides</i>			
(+)-naringenin	272.3	271(151)	27.67

Quantification was achieved by integration of the peak using an external standard method, and results were presented in Table 2.

#### *Tilia argentea* flower with leaves

Lime or linden flowers, *Tiliae flos*, are of prominent importance in phytotherapy. They are stated to possess expectorant, diuretic, diaphoretic, antispasmodic, stomachic and sedative activities and have been used for the treatment of flu, cough, migraine, nervous tension, and ingestion problems, various types of spasms and liver and gall bladder disorders [10-11]. The claimed medicinal properties of the plant have been attributed to its flavonoids, volatile oil and mucilage components [12-13]. The use of the leaves as a remedy is not as common as that of the flowers, but they have been suggested to be employed as a diaphoretic; however, the effect has not been evaluated experimentally so far [14].

In *Tilia argentea* extracts, 70% acetone was found as the most efficient solvent targeting total phenolic content as assessed by LC-ESI/MS and using the Folin-Ciocalteu method. The highest phenolic content in acetone extract could be due to the highest content of protocatechuic acid. The lowest extraction efficiency was found in water extracts. Table 2 shows the recovery of phenolic compounds from different parts of medicinal plants using different solvents.

TABLE II  
QUANTIFICATION OF PHENOLIC COMPOUNDS IN THE EXTRACTS BY LC-ESI/MS

<i>Tilia argentea</i>											
<i>Crataegi filium leaves</i>											
<i>Radii bistoria roots</i>											
Extract	Protocatechuic acid	Gallic acid	Ferulic acid	Coumaric acid	Caffeic acid	Chlorogenic acid	Catechin	Quercetin	Naringenin		
AE	7924±73	N.D	215±10	329±12	6.3±0.2	59±6.8	3306.9±113.1	13.9±1.8	3.1±0.3		
ME	6341±246	N.D	207±2	355±11	5.8±0.3	61.9±2.8	4048.2±84.4	28.9±3.9	7.1±1.2		
WE	1000±33	21	87±5.5	212±4	18.6±1.2	2022±17.5	4179.9±77.9	16.6±1.6	6.6±0.8		
Extract	Protocatechuic acid	Gallic acid	Ferulic acid	Coumaric acid	Caffeic acid	Chlorogenic acid	Catechin	Quercetin	Naringenin		
AE	1346.2±20.4	7.9±0.6	86.4±4.7	225.6±2.9	11.1±1.3	3566.6±97.7	10631.4±75.9	112.7±8.4	7.6±0.2		
ME	975.3±41.3	N.D	48.1±0.7	102.9±4.7	4.6±0.1	1630.5±23.3	5353.6±29.3	43±4.1	3.9±0.3		
WE	1414.4±8.4	92.9±1.6	335.4±17.4	684.4±3.3	12.4±0.5	1251.4±1	284.5±10.1	23.8±5.1	1.7±0.4		
Extract	Protocatechuic acid	Gallic acid	Ferulic acid	Coumaric acid	Caffeic acid	Chlorogenic acid	Catechin	Quercetin	Naringenin		
AE	302.4±3.6	75.2±1.5	3049.9±49.9	446.1±11.2	10.3±0.4	N.D	N.D	3.6±0.3	N.D		
ME	288.5±9.7	57.2±1.5	4.4±1.6	557.3±26.7	N.D	16.1±5.5	N.D	4.4±0.1	N.D		
WE	217.8±11.7	42.8±1.4	45.7±1.3	229.9±13.5	12±0.3	N.D	N.D	45.7±13.1	129.7±22		

Each value is the mean ± standard deviation of triplicate determinations. AE, 70% acetone extract; ME, 70% methanol extract; WE, pure water extract. The concentration is given in µg/g of the dry plant material for triplicate injections.

Catechin, naringenin, quercetin, protocatechuic, p-coumaric, chlorogenic, ferulic, and caffeic acids were determined in all *T. argentea* extracts; however, gallic acid was only found in water extracts. The results indicated that, under our experimental conditions, protocatechuic acid is the major free phenolic compound in acetone (7924  $\mu\text{g/g}$  dw plant) and methanol (6341  $\mu\text{g/g}$  dw plant) extracts of *T. argentea*. Pure water showed the highest extraction capacity for catechin (4179  $\mu\text{g/g}$  dw plant), chlorogenic (2022  $\mu\text{g/g}$  dw plant), caffeic (19  $\mu\text{g/g}$  dw plant) and gallic (21  $\mu\text{g/g}$  dw plant) acids.

For the *T. argentea* extracts, total phenolic content assessed by Folin-Ciocalteu method followed the order: acetone extract > methanol extract > pure water extract (Fig. 1). The values of total phenolic content varied from 5.4 mg GAE/ g dw plant to 18.3 mg GAE/ g dw plant. The data obtained from *T. argentea* extracts demonstrated that various solvent mixtures had significantly different ( $\alpha=0.01$ ) extraction capacities for total phenolic content and antioxidant activity. The antioxidant capacities of *T. argentea* extracts were in the following descending order: acetone extract (14.7 mg AAE/ g dw plant), pure water extract (6.1 mg AAE/ g dw plant) and methanol extract (4.0 mg AAE/ g dw plant). Although the methanol extract had higher total phenolic content than water extract, it did not exhibit higher antioxidant activity than the water extract.

#### *Crataegi folium* (Hawthorn) leaves

In phytomedicine, the applicable parts of Hawthorn (*Crataegi folium*) are the fruit, leaf and flower. Extracts of the leaf and flower are the most commonly used for therapy. In the Hawthorn leaf and flower, the active constituents mostly important for the therapeutic action are the flavonoids, phenolic acids and oligomeric proanthocyanidins. Hawthorn leaf is used orally for cardiovascular conditions such as congestive heart failure, coronary circulation problems, and arrhythmias. It is also used to increase cardiac output reduced by hypertension or pulmonary disease, to support hypotension and hypertension, atherosclerosis, hyperlipidemia and Buerger's disease. Topically, Hawthorn leaf is used as a poultice for boils, sores and ulcers [15].

The catechin content in the Hawthorn leaves ranged from 284 to 10631 in  $\mu\text{g/g}$  of dry weight of leaves. The highest catechin content was found in 70% acetone extract. In acetone and methanol extracts, protocatechuic acid was the second prominent phenolic compound. In methanol extract, gallic acid was not detected. In the extracts, naringenin had the lowest content. The recovery yield for the identified phenolic compounds was highest in acetone extract and lowest in water extract.

In Fig. 1 the total phenolic contents obtained from Hawthorn leaves are presented. Among all the different solvent extractions, acetone extract had the highest phenolic content (36.6 mg GAE/ g dw leaves), followed by methanol extract (12.3 mg GAE/ g dw leaves) and pure water extract (11.1 mg GAE/ g dw leaves). Water extract of Hawthorn leaves exhibited the highest antioxidant activity (11.5 mg AAE/ g dw

leaves), followed by acetone extract (8.7 mg AAE/ g dw leaves) and methanol extract (3.8 mg AAE/ g dw leaves) (Fig. 2). Antioxidant capacities of aqueous extracts of acetone, methanol and pure water were significantly different ( $\alpha=0.01$ ). Water extract had the highest contents of protocatechuic, ferulic, coumaric, caffeic and gallic acids. For this reason, it could exhibit higher antioxidant activity compared to other extracts.

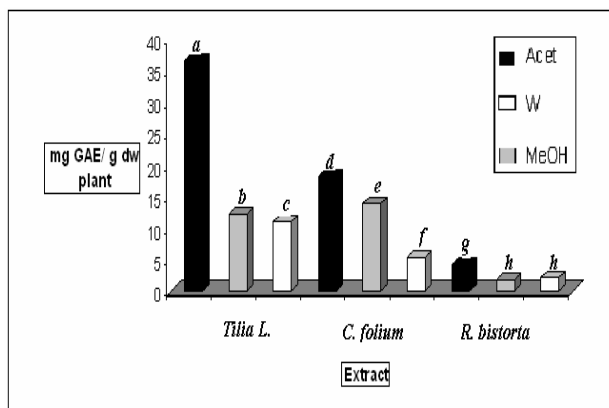


Fig.1 Total phenolic contents of different solvent extracts. The concentrations of all tested extracts are on the same dry weight basis. Significantly different ( $p<0.01$ ) extracts are marked by different letters.

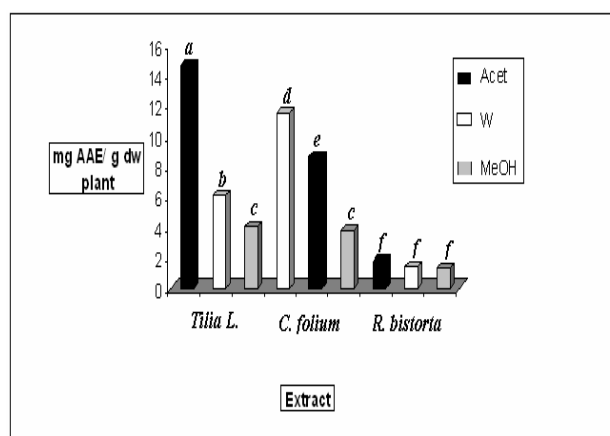


Fig.2 ABTS radical cation scavenging activities of different solvent extracts. The concentrations of all tested extracts are on the same dry weight basis. Significantly different ( $p<0.01$ ) extracts are marked by different letters.

#### *Polygonum (Radix) bistorta* roots

*Polygonum bistorta*, with its common name, *Radix bistorta* is classified in Polygonaceae family. The roots of *Polygonum bistorta* are rich in starch and tannin. The root could be used in soups and stews and it can be dried and ground into a powder and used in bread making. The root is powerfully astringent, demulcent, diuretic, febrifuge, laxative and styptic. It is gathered in early spring when the leaves are just beginning to

shoot, and then dried [16]. It is used, both internally and externally, in the treatment of bleeding, diarrhea, dysentery and cholera [17]. In addition, it could be used internally in the treatment of a wide range of complaints including catarrh, cystitis, irritable bowel syndrome, peptic ulcers, ulcerative colitis and excessive menstruation [18].

The amount of total phenolics in *R. bistorta* roots ranged between 2336 to 3887  $\mu\text{g/g}$  dw plant material according to the LC-MS data. The minimum and maximum extraction rates were acquired by pure water and 70% acetone solvent respectively. The LC-MS profiles of the extracts demonstrated protocatechuic, ferulic, coumaric and gallic acids as major phenolic compounds. Catechin was not found in the extracts. Naringenin and chlorogenic acid was only found in water and methanol extracts respectively. 70% acetone extract had the highest phenolic content (4.19) followed by 70% methanol extract (2.24) and pure water extract (1.92) in mg gallic acid equivalents per g dry weight of plant material. There was no significant difference between methanol and water extracts ( $\alpha=0.01$ ). Radical scavenging activities of extracts did not show any significant differences within each other ( $\alpha=0.01$ ). The antioxidant capacities and total phenolic contents of *R. bistorta* roots were the lowest among other extractions prepared by Hawthorn leaves and *T. argentea*.

#### IV. DISCUSSION

Three kinds of extracts with 70% acetone, 70% methanol and pure water were prepared from each plant material. The extraction yield of antioxidant compounds from plant materials is influenced mainly by the conditions under which the process of liquid-solid extraction is achieved, the type of the solvent used to separate the soluble fraction from the permeable solid, the degree of polymerization of phenolics and their interaction with the other components [19]. The selection of an appropriate solvent is one of the most relevant previous steps in this operation. In this study, the highest extraction efficiency was found in 70% acetone extracts while the lowest extraction efficiency was found in water extracts of medicinal plants due to the total phenolic content by LC-ESI/MS and Folin-Ciocalteu assay. The reason for the high extraction efficiency with aqueous solvents could be primarily due to the water-soluble nature of plant phenolics enhanced by the presence of an organic solvent, which facilitates solubilization through penetration in plant cell structure [20].

Based on the optimized conditions, nine phenolic compounds, were identified in varying concentrations, are representative of diverse structural types that might contribute to the antioxidant behavior of the extracts. Under our experimental conditions, catechin was the major free phenolic compound detected in Hawthorn leaves' and *T. argentea* extracts with the exception of water extract of Hawthorn leaves. In water extract of Hawthorn leaves, protocatechuic acid was found as the major phenolic compound. In addition, catechin was not detected in *R. bistorta* roots. On the other hand, protocatechuic, ferulic and p-coumaric acids were also

identified. Chlorogenic was detected in all plant extracts with exception of methanol and water extracts of *R. bistorta* roots. In addition to five phenolic compounds, quercetin, naringenin, caffeic and gallic acids found as minor compounds in all the extracts.

The LC-ESI-MS data and the results obtained by Folin-Ciocalteu assay showed a positive correlation ( $R^2=0.78$ ). Total phenolic content obtained by Folin-Ciocalteu assay in the medicinal plants are significantly different except for 70% methanol and water extracts in *R. bistorta* roots ( $\alpha=0.01$ ). Significant differences between the plants are likely due to genotypic and environmental differences (climate, location, temperature, fertility, diseases and pest exposure) within species, choice of parts tested, time of taking samples and determination methods. In plants, polyphenols synthesis and accumulation is generally stimulated in response to biotic/abiotic stresses (salinity, temperature, water stress and light intensity) [21]. Previous studies indicated that secondary metabolites' distribution might fluctuate between different plant organs [22].

Typical phenolics that possess antioxidant activity are known to be mainly phenolic acids and flavonoids. Phenolic acids are a major class of phenolic compounds, widely occurring in the plant kingdom especially in fruits and vegetables. Many medical herbs and spices have been studied and to some extent, their phenolic chemistry is known [23]. Total phenolic compounds determined by LC-ESI-MS and Folin-Ciocalteu assay showed a poor correlation with antioxidant activity of plant extracts. Although the acetone extracts of Hawthorn leaves showed the highest total phenolic content, they did not exhibit the highest antioxidant activity whereas 70% acetone extracts of *T. argentea* exhibited the highest antioxidant activity. As discussed above, the difference in antioxidant activities of the extracts could be due to the different polarities of the solvents, and thus different extractability of the antioxidative compounds [24]. Antioxidant properties of single compounds within a group can vary remarkably, so that the same levels of phenolics do not necessarily correspond to the same antioxidant responses [25]. Different reports are found on the literature; whereas some authors found correlation between the total phenolic content and the antioxidant activity, others found no such relationship. For instance, Velioglu et al. [26] reported a strong relationship between total phenolic content and total antioxidant activity in selected fruits, vegetables and grain products, whereas Kahkonen et al. [4] found no correlation on the same plant extracts containing phenolic compounds. In addition, in another study with citrus residues, no correlation was found [27]. There is a wide degree of variation between different phenolic compounds in their effectiveness as antioxidants. The different antioxidant activities of phenolic extracts rich in phenolic compounds can be attributed to different extracting solvent as the antioxidant activity depends on the type and polarity of the extracting solvent, the isolation procedures, the purity of active compounds, as well as the test system [28]. Determination of an absolute value for the antioxidant capacity of an extract is difficult because it depends on the actual

concentration of the radical, its degradation during analysis, or matrix interference [29].

## V. CONCLUSION

Our results showed that Turkish species of Hawthorn leaves and *T. argentea* were rich in phenolic constituents and demonstrated good antioxidant activity, whereas *Radix bistorta* root extracts were found to be poor in antioxidant capacity measured by ABTS assay. The chromatographic separation enabled the identification of a wide range of phenolic compounds present in medicinal plants without time-consuming sample preparation or previous fractionation. These plants, rich in flavonoids and phenolic acids could be a good source of natural antioxidants. Therefore, the qualitative and quantitative analysis of major individual phenolics in the species could be helpful for explaining the relationships between total antioxidant activity and total phenolic content of the extracts. Obviously, to confirm the beneficial effects of these extracts, it is necessary to carry out further studies about their in vivo activity and bioavailability.

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