In Vitro Antioxidant Activities of Methanol Extracts of Three *Achillea* Species from Turkey

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Abstract: In this study, total phenolics and flavonoid compounds of methanolic extract concentrations of three medicinal plant, Asteraceae (Compositae) L. genus that are grown in Turkey and belong to the genus Achillea, were determined with the goal of measuring their antioxidant activities. Antioxidant capacity was measured by widely used iron reducing power, DPPH radical scavenging activity and metal chelating capacity. The antioxidant activities of the achillea extracts used in the study were compared with the standard antioxidants (BHA, BHT and α-tocopherol), which were frequently used as antioxidant food additives. According to the free radical scavenging activity antioxidant results, all the extracts exhibited higher DPPH radical scavenging activity than the standards used. The extract from *A.boissieri* showed remarkable 2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity (68.51% at 37.5 µg/mL) comparable with synthetic antioxidants. The ferric reducing antioxidant power was found to be moderate in methanolic extract of Achillea species, whereas the chelating capacity of the extracts were found to be lower as compared to the standards. In addition, total phenolic, flavonoid and flavonol content of all extracts were measured spectrophotometrically and the results were expressed as "gallic acid equivalent" or "quercetin equivalent". The total phenolic content was expressed as equivalents of gallic acid and the results were observed to range from 11.86 to 23.63 mg/g dry extract weight. The total flavonoid concentrations of Achillea extracts were expressed as quercetin equivalent. Flavonoid content ranged from 15.05 to 29.70 mg/g. Total flavonol concentrations of the extracts were determined to be between 5.92 and 7.20 mg/g in terms of quercetin equivalent. This study showed that Achillea L. species, which has been used for treatment in Anatolia for years, can be used as a potential natural antioxidant source.

Keywords: Phenolic content, flavonoid content, antioxidant activity, *Achillea L.* species

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INTRODUCTION

A free radical can be defined as any chemical species that are capable of existing with one or more unpaired outer shell electrons (1). Their high chemical reactivity due to the presence of an unpaired electron makes them highly unstable (2) and in an attempt to reach stability. The unpaired electron in a free radical can either donate an electron to or receive an electron from other molecules such as proteins, lipids, carbohydrates, and nucleic acids. This process causes damage to cell and tissue or various diseases from cardiovascular diseases to the promotion of cancer (3,4). Antioxidant compounds act by controlling oxidative stress through different reaction mechanisms and may prevent the occurrence of these diseases (5).

A variety of plant materials are potential sources of natural antioxidants. A plant-based antioxidants may support the antioxidative defense (6-8). The

genus *Achillea* L., comprising about 120 species, is mainly spread over the northern hemisphere. The species occur throughout Europe, Asia, China and North Africa, however its center of diversity is located in SE Europe and SW Asia. (9,10) Many *Achillea* L. species are used for the various ailment in Turkish folk medicine due to their high nutritional value and valuable biological activities (11,12).

The fact that Achillea species have a mythological history, the number of endemic species is quite high, their use among the people is widespread, and their pharmacological effects have prompted us to study these species. In our study, it was aimed to elucidate the chemical structures of the aerial parts of *Achillea cretica, Achillea boissieri* and *Achillea nobilis* subsp. *spiylea* collected from different locations of Turkey, which are widely used among the public, and to investigate the antioxidant effects of the species. Radical scavenging of 2,2-diphenyl-1-picryl-hydrazyl (DPPH), metal chelating power and ferric reducing power assays were used to measure the antioxidant capacities of the extracts.

EXPERIMENTAL SECTION

Chemicals

All reagents were purchased from Merck (Darmstadt, Germany), Sigma–Aldrich (Steinheim, Germany), Acros Organics (Thermo Fisher Scientific, Reel, Belgium) and Fisher Scientific (Hampton, NH), while all solvents used were of analytical grade.

Plant materials and extracts preparation

A. boissieri was collected in August 2007 at the flowering period from Elbistan, between Kabaktepe-Sariguzel, 1600 m altitude from Kahramanmaras Province (Turkey). The voucher specimen have been deposited in the Herbarium of Inonu University (INU) in Malatya, Turkey (INU-Collector No: TA 2594).

A. cretica L. (Asteraceae) was collected in June 2004 at the flowering period from Datça-Knidos, Muğla province, Turkey (2100 m above sea level). The voucher specimen has been deposited in the Herbarium of Inönü University (INU) in Malatya, Turkey (INU-Collector No: BY 15634).

A. nobilis subsp. *spiylea* was collected in June 2004 at the flowering period from Spil Mountain, 1100 m altitude from Manisa Province (Turkey). The voucher specimen has been deposited in the Herbarium of Inonu University (INU) in Malatya, Turkey (INU-Collector No: BY 15750).

The aerial parts of the three *Achillea* L. species were dried in shade for 7 days with occasional mixing and cut into small pieces. The dried stock samples were kept in airtight containers at 4 °C for future extraction. After taking 20.000 ± 0.001 g of dried plant samples prepared as described above, it was extracted with methanol using the Soxhlet extraction method until it was completely exhausted. The methanol extracts were concentrated using a rotary evaporator (Heidolph Laborota 4000, Heidolph Instruments, GmbH and Co, Germany) at 40 °C to obtain a viscous liquid. The concentrated extract was transferred to a 25 mL beaker and the remaining solvent was evaporated in the laboratory. The extracts dried after evaporation were weighed to calculate the yield and were stored at +4 ºC in a refrigerator until further analyses. The percentage yield for the extracts was as given in Table 1.

Total phenolic content (TPC)

The content of total phenolics of *Achillea* L. extracts was determined using Folin-Ciocalteu's reagent according to the method of Singleton et al. (13). The absorbance of reaction mixtures was measured at 765 nm (Shimadzu model UV-1601, Japan). The total amount of phenolic substance was calculated from the standard calibration curve prepared using the gallic acid standard, which is a phenolic compound. The results are expressed as mg GAE/g extracts on the gallic acid equivalent by using the regression equation of the curve obtained. Spectrophotometric measurements were repeated three times for each sample, and the total phenolic content was indicated by taking the average of triplicate measurements.

Total flavonoid and flavonols content

The content of total flavonoids of extracts was determined according to the procedure described by Zhishen et al. (1999) (14). Total flavonoid content of the extracts was determined spectrophotometrically according to the aluminum chloride/sodium nitrite method. Total flavonol content was determined by the method described by Yermakov et al. (1987) with minor modifications (15). Briefly, 1 mL of extract was mixed with 1 mL of $AlCl_3$ (%2) and 3 mL of sodium acetate (50 g/L). After 150 min the absorbance of the test solution was measured at 440 nm against blank solution. Total flavonoid/flavonol content of the extracts in certain concentration ranges was calculated according to the quercetin standard curve prepared by working in triplicate, results were expressed as mg of quercetin equivalent per gram dry extract.

Antioxidant Activities of *Achillea* Extracts

Antioxidant assay by DPPH radical scavenging activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity of *Achillea* L. extracts was performed according to methodology described by Blois (1958)(16). This method involves the reduction of the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical with compounds that tend to give hydrogen atoms, resulting in the loss of purple color of the solution at the first moment and this ratio is based on spectrophotometric measurement of the absorbance of the solution. BHA, BHT and alpha-tocopherol were used as standards. As a control, ethanol was used. The absorbance was measured at 517 nm and the

absorbance values of the samples were evaluated against the control. Free radical scavenging activity was calculated using the following equation:

$$\% \text{ Inhibition} = (A_B - A_{S/S}) / A_B \times 100$$

A_B is the absorbance of control and $A_{S/S}$ is the absorbance of the analyzed standard/sample.

Ferric-reducing antioxidant power assay

The reducing power, which is one of the antioxidant activity determination methods, was determined based on the method applied by Oyaizu (1986) (17). In this experiment, the yellow color turns pale green and blue, depending on the antioxidant concentration in the samples. The color produced by the reduction of $Fe^{3+} \rightarrow Fe^{2+}$ the change is determined by monitoring at 700 nm. Increased absorbance of the mixture indicates stronger reducing influence of the extract.

Metal chelating activity

Metal chelating activity was determined by measuring the formation of the Fe^{2+}-ferrozine complex according to Carter (1971)(18). The Fe(III)-ferrozine method is based on the principle that Fe(III) forms the complex of ferrozine and Fe(III)-ferrozine, and this complex reacts with antioxidants and is reduced to the magenta-colored Fe(II)-ferrozine complex (absorption maximum at 562 nm) (19). Therefore, the change in color was measured using a spectrophotometer against blank at 562 nm. The percentage inhibition of ferrozine–Fe^{2+} complex formation was calculated as $(A_{control} - A_{sample/standard})/A_{control}$ x 100, where $A_{control}$ is the absorbance of control reaction (without analyzed sample extract), and A_{sample}/ $A_{standard}$ is the absorbance of the analyzed sample/standard. The values are presented as the mean of three measurements.

RESULTS AND DISCUSSION

The medicinal plants are huge natural sources of secondary compounds with health-promoting properties. The polyphenols derived from plants have significant antioxidant effects, which allow them to provide health benefits. Epidemiological evidence and clinical trial data obtained from in vivo and in vitro studies have shown that diets based on herbal products rich in polyphenols can reduce the risk of chronic diseases, especially cancer. Polyphenolic substances, which are named according to the number of rings and structural elements they contain, generally have a phenol ring as in phenolic acid and alcohols. More than 8000 types of polyphenols have so far been identified in nature. The four most important groups of dietary phenolics are flavonoids, phenolic acids, polyphenolic amides and other polyphenols such as curcumin in turmeric, resveratrol in red wine and 5-caffeoylquinic acid in black carrot roots. Flavonoids are the largest group of plant phenols, with more than 6000 types. There are several significant groups of flavonoids, including flavonol, flavanol, isoflavon, flavon, flavanon and anthocyanin (20-22).

Phenolic acid constituents in plant kingdom are mainly divided into hydroxybenzoic acid and hydroxycinnamic acid. These compounds possess much higher in vitro antioxidant activity than well-known antioxidant vitamins, although some of them are also regarded as anticarcinogenic (23,24). Polyphenolic amides include capsaicinoids in chili peppers and avenanthramides in oats (25,26). Both animal studies and laboratory studies have confirmed that phenolic amides have antioxidant activities (27-29). The species included in the genus *Achillea* L., which contain important bioactive components, have been used for therapeutic purposes in many parts of the world for centuries.

This study focused primarily on the determination of antioxidant activity of methanolic extract of Turkish *Achillea cretica, Achillea boissieri* and *Achillea nobilis* subsp. *spiylea* by *in vitro* methods. We measured the phenolic, flavonoid and flavonol contents in the *A.cretica*, *A. boissieri* and *A. nobilis* subsp. *spiyle*a extracts we obtained.

Table 1. Analysis of main antioxidant fractions contained in *A. cretica, A. boissieri* and *A. nobilis subsp. spiylea* extracts.

Samples	Yield (%)	Phenolics (mg GAE/g plant extract)	Flavonoids (mg QUE/g plant extract)	Flavonols (mg QUE/g plant extract)
A. cretica	0.807 ± 0.170	11.86 ± 0.09	15.05 ± 0.17	15.92 ± 0.11
A. boissieri	5.375 ± 0.810	23,63 ± 0.17	29.70 ± 0.03	17.20 ± 0.19
A.nobilis* subsp. *spiylea	7.114 ± 1.200	17.33 ± 0.09	18.20 ± 0.03	16.95 ± 0.04

Each value is the mean ± SD of three independent measurements. Phenolics, gallic acid equivalents; flavonoids and flavonols, quercetin equivalents.

The results (Table 1) showed that the *A. boissieri* extract exhibited higher total phenolics content as compared to the *A. cretica* and *A. nobilis subsp*. s*piylea* extracts which were approximately about 23.63 mg GAE/g for *A. boissieri* extract, 11.86 mg GAE/g for *A. cretica* extract, and 17.33 mg GAE/g for *A. nobilis subsp*. s*piylea* extract. The contents of flavonoids and flavonols were also higher in A. boissieri extract than in *A. cretica* and *A. nobilis* subsp. *spiylea*. This result clearly indicates that *A.*

boissieri extract contains more antioxidants than the *A. cretica* and *A. nobilis subsp*. *spiylea* extract.

Anti-oxidant effects of plant extracts

The DPPH radical scavenging activity results are shown in Figure 1 as comparable with known antioxidants BHT and BHA. From the analysis of Figure 1, we can conclude that the scavenging effects of *A. cretica, A. boissieri* and *A. nobilis subsp*. *spiylea* extracts on DPPH radicals were excellent, especially in the case of A. boissieri. The antioxidative effect of extracts studied is due to the phenolic components. Similar results were obtained earlier for the species of Achillea L. from Turkey (30,31).

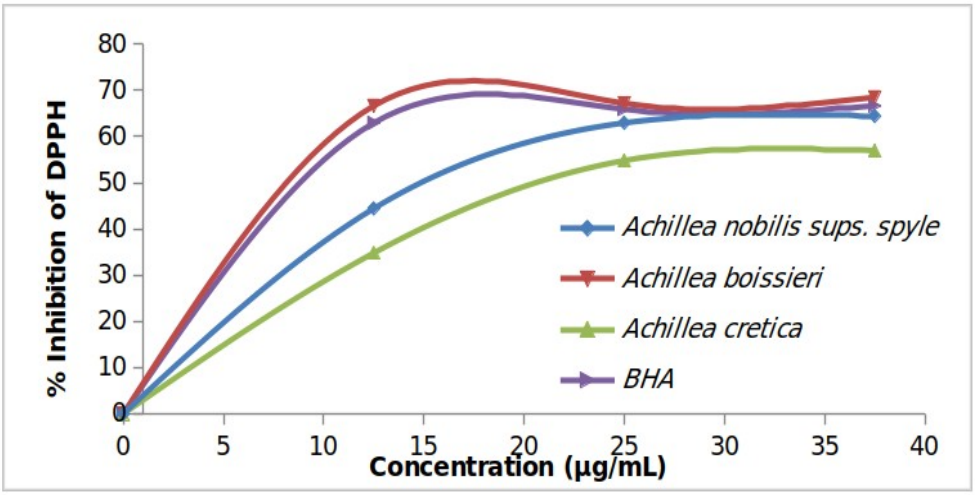

Figure 1 . Dose-dependent scavenging activity of the extracts and the standard BHA, BHT, and alpha-tocopherol on 1,1-diphenyl-2-picrylhydrazyl inhibition.

As described above, the antioxidants such as phenolic acids and flavonoids were present in considerable amount in the extracts of *A. cretica, A. boissieri* and *A. nobilis* subsp. *spiylea*. The prepared methanol extracts of *A. cretica, A. boissieri* and *A. nobilis* subsp. *spiylea* were compared with the standard BHA, BHT and alpha-tocopherol antioxidants. Like the radical scavenging activity, the reducing power of the extracts and standard increase with the increase in amount of the extracts from the selected plants and standard concentrations (Figure 2). At the minimum concentration of extract/standards used in this study (i.e. 5.88 µg/mL), *A. boissieri, A. nobilis* subsp. *spiylea, A. cretica* and butylated hydroxyanisole (BHA) had activity values 0.111 ± 0.001, 0.102 ± 0.001, 0.098 ± 0.001 and 0.630 ± 0.001, respectively whereas at the highest concentration (i.e. 44.11 µg/mL), the activity values of *A. boissieri, A. nobilis* subsp. *spiylea, A. cretica* and butylated hydroxyanisole (BHA) were 0.343 ± 0.001, 0.229 ± 0.001, 0.167 ± 0.002 and 2.108 ± 0.003, respectively.

Metal chelating activities of standard antioxidants and extracts were tested at various concentrations (12.5-125 µg/mL). Calculated % inhibition values are given in Figure 3. The higher the calculated % inhibition value, the higher the metal chelate activity was accepted and the results were evaluated. EDTA-Na_2 was excellent chelator for ferrous ions and its chelating capacity was 94.311% at a concentration of 125 µg/mL. BHA, alpha-tocopherol, *A. cretica* and *A. nobilis* subsp. *spiylea* did not show chelating capacity at all concentrations as well. This proves that these extracts and reference compounds have a lower capacity to chelate them with ferrous ions compared to the standard chelator EDTA. In a previous study with Achillea species, *Achillea aleppica* D.C. subsp. *aleppica*, *Achillea aleppica* D.C. subsp. *zederbaueri* (Hayek) Hub.-Mor and *Achillea biebersteinii* Afan. species that have compared antioxidant activity, antimicrobial activity and total phenolic amounts, it has been reported that *Achillea biebersteinii* Afan. species were richer than other species in terms of total phenolic. Also, Barış et al. (2011) used EDTA solution as the standard chelator in their study with Achillea species. They found that extracts in this system were not a better chelator than EDTA solution, which was a good chelator (32).

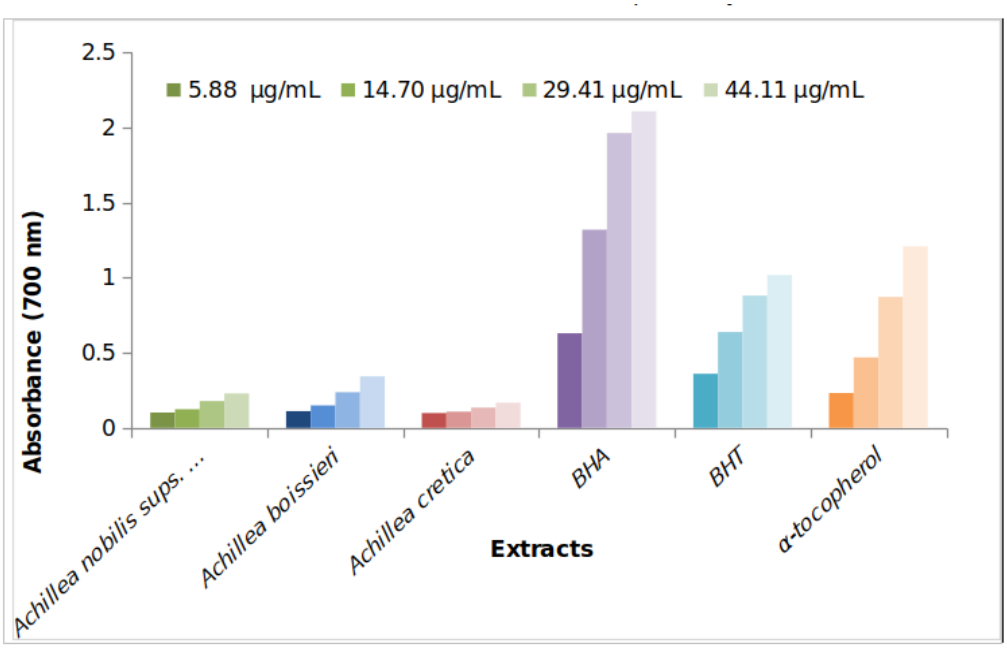

Figure 2. The reducing power of extracts and reference compounds. Values were reported as means ± SD in triplicate.

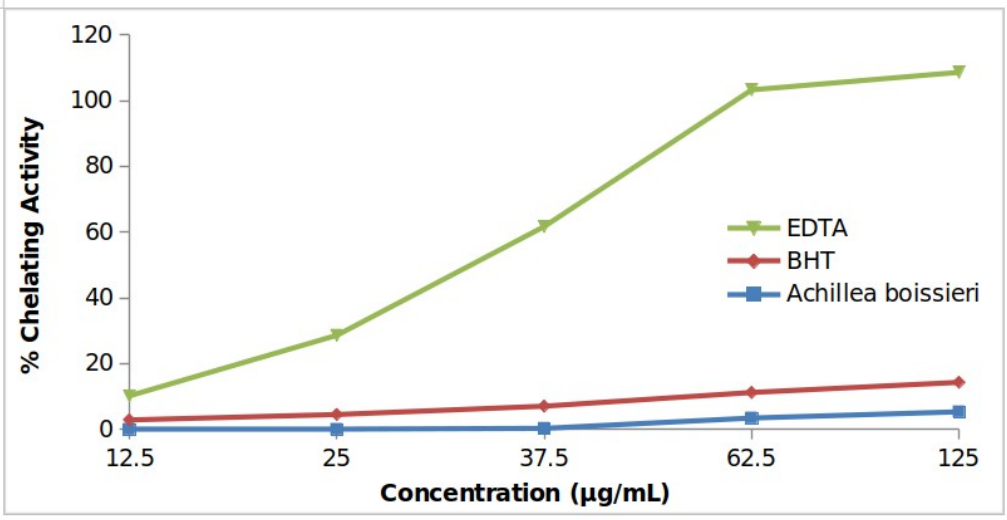

Figure 3. Metal chelating activities of *Achillea* extracts at different concentrations. Data are expressed as means ± SD values (n=3).

CONCLUSIONS

Achillea species have been preferred as folk remedies for various purposes for a long time. Therefore, the species named *A. boissieri, A. nobilis* subsp. *spiylea* and *A. cretica*, which spread in Turkey, were investigated in terms of antioxidant activity and amounts of phenolic/flavonoid/flavonol compound. As a result of the literature studies, no antioxidant properties were evaluated for these 3 species used in our research. Especially, the DPPH radical scavenging activities of *A. boissieri* methanol extract was determined to be higher compared to the standard used BHA and can be used as a herbal antioxidant. It is believed that the results of this study will contribute to increasing studies on the use of natural compounds in many fields, especially in food, pharmacy, medicine and natural therapy. The fact that the biological properties of the studied plant were investigated for the first time with this study increases the original value of the study. As a result, the studies conducted are very original studies in terms of this species, which has not been found before. In the following studies, isolation, purification and clarification of the structure of

active compounds with bioactivity can be carried out.

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Conflicts of interest
There is no potential or existing conflict of interest between our scientific work and our personal situation.

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