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Determination of Antioxidant Properties of *Gypsophila bitlisensis* Bark.

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ABSTRACT
Oxidative stress has been suggested to explain the mechanism of numerous diseases and antioxidants have been proposed to prevent cellular oxidative damage, therefore disease progression. Antioxidant capacity of many plants, fruits and vegetables have been studied by the researches, however there was no data regarding the antioxidant properties of *Gypsophila bitlisensis*. The species is an endemic and natural plant of Turkey. We performed three assays to reveal antioxidant capacity of ethanol extract of *Gypsophila bitlisensis*: FRAP, DPPH and CUPRAC. Trolox and α-tocopherol have been used as standards. We also determined phenolic content of the extract. The results showed that *Gypsophila bitlisensis* does not have significant antioxidant capacity and phenolic content. On the basis of these findings, we concluded that *Gypsophila* does not have considerable antioxidant activity in these *in vitro* assays.

Key words: Antioxidant, *Gypsophila bitlisensis*, oxidative stress, FRAP, DPPH, CUPRAC

INTRODUCTION

Under normal physiological conditions, Reactive Oxygen Species (ROS) are produced. These species play important role in signalling, cellular redox cycling and activation of some enzymes. The excess ROS are removed from the cell, as they are also capable of oxidizing biomacromolecules. If ROS are not efficiently removed by antioxidants defences (enzymatic, non-enzymatic or dietary origin) oxidative stress occurs (Bursal and Gulcin, 2011). Chronic oxidative stress results in oxidative modification of proteins, lipids and nucleic acid which can initiate abnormal tissue functions and diseases. Consumption of foods containing natural antioxidants can be used to eliminate the damage caused by ROS and reduce oxidative stress related impairments. Therefore, assessing the antioxidant capacity of the food has become one of the interests in the research area (Bursal and Gulcin, 2011).

Generally, mitochondria and cytochrome 450 enzymes are the main producers of ROS. The factors contributing ROS formation could be endogenous such as pathophysiology, exercise, drug metabolites and ion influx, or exogenous such as drugs, toxicants, pathogens and dietary ingredients. The primary free radicals in the cells are superoxide ($O_2^{\bullet -}$) and Nitric Oxide (NO). The $O_2^{\bullet -}$ is generated as a consequences of incomplete reduction of oxygen in electron transport systems or by specific enzymatic reactions whereas NO is the product of some specific enzymes (the nitric oxide synthases). There are also nonradical oxygen species like H_2O_2. This is particularly interesting since it is neutral, membrane permeable and can oxidise thiol of cysteine residues which alter the activity of target protein and has role in signalling pathways. The H_2O_2 can also give rise to $OH^{\bullet}$ radical via Fenton reaction in the presence of metal ions. Hydroxyl radicals also form due to indirect effect of X- and gamma-rays through the radiolysis of water (Von Sonntag, 2006).

There are numerous studies showing the direct effect of ROS on disease progression. Perhaps the most accepted idea of involvement of ROS in human disease and toxicology comes from the detection and determination of lipid peroxidation (Valko *et al*., 2004). For example malondialdehyde is a chain cleavage product of peroxyl radicals and it was elevated in patients with liver cancer (Arslan *et al*., 2014). Attack of peroxyl radicals may result in DNA cleavage and protein backbone modification (Valko *et al*., 2004). In addition, H_2O_2 has been linked to the formation and accumulation of amyloid beta in brain and retina cells which results in lens opacification (Melov *et al*., 2005). The H_2O_2 has been also suggested to be generated in early stages of aggregate formation in Alzheimer diseases (Tabner *et al*., 2005). On the other hand, hydroxyl radical is also harmful to living cell by causing damage in DNA. Strand breaks, complex DNA lesions, protein-DNA cross-links and protein and DNA adducts have been suggested to be results of hydroxyl radical induced damage (Cadet and Wagner, 2014).

Despite detrimental effect of ROS, cell has antioxidant defence system which removes ROS and therefore help preventing cancer, heart diseases and stroke (Valko *et al*., 2004). It has been reported that consumption of vegetables is related to reduced risk of digestive tract neoplasms and fruits is related to reduced risk of cancers of the upper digestive tract, stomach and urinary tract (La Vecchia *et al*., 2001) Moreover, dietary intake of antioxidants has been shown to have positive impact on immune system, cardiovascular diseases, cataracts, as well as brain dysfunction (Ames *et al*., 1993).

Studies showing the beneficial effects of specific antioxidants are also accumulating. For example N-acetylcysteine, a thiol containing antioxidant, has been shown to protect rats from memory impairment after sepsis induction (Barichello *et al*., 2007). Also a recent study has shown that early induction of vitamin E and lycopene suppressed allergenic airway disease in the later life in mice (Hansbro *et al*., 2014). Consumption of food rich in vitamin E and C has been related to delayed development of the various forms of cataracts (Jacques *et al*., 1994).

Numerous foods have been reported as source of natural antioxidants and inadequate consumption of these foods has been related to increased risk of cancer (Block *et al*., 1992). Thus discovery of novel species is useful in terms of preventing diseases and delaying aging. *Gypsophila* L. (family: Caryophyllaceae), are annual, biennial or perennial suffrutescent herbs with linear-subulate leaves which mainly gown in Mediterranean zone (Yucekutlu and Bildaci, 2008). There are around 150 *Gypsophila* species which are commonly used in the industry and medicine to treat some diseases such as hepatitis, gastritis and bronchitis and also used as expectorant (Simeonova *et al*., 2013; Yucekutlu and Bildaci, 2008). *Gypsophila* is the third biggest genus of Caryophyllaceae family in Turkey. The genus has 55 species in the country and 33 species of them are endemics. All members of the genera are known as Coven and developed roots are economically very important because of their different amount of saponin contents. Extract produced from their roots are known as fire extinguisher, gold polishing, cleaner and softener of delicate fabrics and crispness giving to halva. The extracts are often used for making liqueur, herbal cheese, ice cream and different foods. Some species of the genus are Boron (B) hyperaccumulators (Korkmaz and Ozcelik, 2011). *Gypsophila* has shown to have moderate antimicrobial activity and substantial effect on fungi (Shafagha and Shafaghatlonbar, 2011). The presence of triterpene saponins (Arslan *et al*., 2013), sterols, flavonoids (Darmograi *et al*., 1969), triterpens in different *Gypsophila* species (Krasteva *et al*., 2014) were indicated. *Gypsophila* saponins have been also reported to have *in vitro* cytoprotective effect on galactosamine induced hepotoxicity (Braut Boucher *et al*., 1990). It was proposed that *Gypsophila* might have anticancer activity due to containing an active component called gypsogenin at high concentrations (Emirdag-Ozturk *et al*., 2014). In addition, antioxidant effect of saponarin isolated from *Gypsophila trichotoma* was determined, however, there is no report on overall *in vitro* antioxidant properties of the genus *Gypsophila*. Therefore, it was considered useful to determine the antioxidant properties of *Gypsophila bitlisensis* in order to provide reference to the consumers.

MATERIALS AND METHODS

Chemicals: The 2,9-dimethyl-1,10-phenanthroline (neocuproine), the stable free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH•), 3-(2-Pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine®), 6-hydroxy-2, 5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), α-tocopherol, $CuCl_2$, Folin-Ciocalteu reagent and gallic acid were obtained from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). All other chemicals used were analytical grade and obtained from Merck.

Sample preparation: This plant material was collected between Büyükyayla and Mezra villages in a wet habitat from Çayırlı-Erzincan, Turkey in 2011. The plant samples of the species were identified and some of them were deposited in Erzincan University Herbarium with the sample number of Korkmaz: 2881.

For Ethanol Extract of *Gypsophila* (EEG), about 25 g of *Gypsophila bitlisensis* was grounded to get fine powder and then mixed with 100 mL ethyl alcohol on a magnetic stirring for 1 h. Remaining residue was re-extracted until colourless extraction solvent was obtained (final volume of 600 mL). After extracting the sample through Whatman No. 1 paper, ethanol was removed using a rotary evaporator (RE 100 Bibby, Stone Staffordshire England) at 50°C to obtain dry extract. The extracts were stored at -20°C in dark plastic bottle until experimental studies.

Ferric cyanide (Fe^{3+}) reducing antioxidant power assay (FRAP): The FRAP assay was performed as previously described (Bursal and Koksal, 2011). The method is based on the spectrophotometric measurement of complex formed when

ferric tripyridyl triazene (Fe^{3+}-TPTZ) complex was reduced to the ferrous (Fe^{2+}) ion at 700 nm. Briefly, different concentrations of the samples (10, 20 and 30 μg mL^{-1}) in 0.75 mL water was mixed with 1 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 1 mL (1%) of potassium ferricyanide (1%). After incubating the mixture at 50°C for 20 min, trichloroacetic acid (10%) was added to mixture to acidify. Absorbance was measured by using distilled water as blank and for control. Higher absorbance indicates better reducing capacity of the sample.

Cupric ions reducing assay (CUPRAC): The protocol for determining the capacity of reducing power: The capacity of sample to reduce cupric ions (Cu^{2+}) was determined by CUPRAC assay as previously described (Bursal and Koksal, 2011). A volume of 0.25 mL $CuCl_2$ (0.01 M), 0.25 mL neocuproine (7.5 mM) in ethanol and 0.25 mL NH_4Ac (1 M) was mixed with standards and sample solutions at different concentrations (10, 20 and 30 μg mL^{-1}). Total volume was adjusted to 2 mL with distilled water and the mixture was kept in the room temperature for 30 min. The absorbance values were read at 450 nm against blank.

1, 1-diphenyl-2-picryl-hydrazil (DPPH) free radical scavenging activity: The DPPH free radical scavenging activities of *Gypsophila bitlisensis* was measured according to the method described by Blois (1958). In the method, the purple colour exhibited by the stable DPPH radical is bleached by free radical scavenging activity of the sample which could be monitored at an absorbance of 517 nm. Briefly, different concentrations (10, 20 and 30 μg mL^{-1}) of ethanol extract from *Gypsophila bitlisensis* were prepared and volume adjusted to 3 mL with ethanol. Alcoholic solution of DPPH (1 mL, 0.1 M) was added to sample, followed by incubation in the dark for 30 min. A reduction in the absorbance indicates DPPH• scavenging activity.

Determination of total phenolic content: Total phenolic analysis was performed using Folin-Ciocalteu reagent as previously described (Bursal and Koksal, 2011). The sample test (1 mg) was mixed with 23 mL water, then Folin-Ciocalteu phenol reagent (0.5 mL) and 3 min later, 2% Na_2CO_3 (1.5 mL) were added. The mixture was vortexed and kept in the room temperature for 30 min. Absorbance of sample was measured at 760 nm. Gallic acid was used as standard phenolic compound and calculation was made on the basis of a standard curve of gallic acid. The phenolic content of *Gypsophila bitlisensis* was calculated by using a standard curve and expressed as s gallic acid equivalents per mg extract.

Statistical analysis: The results were expressed as the Mean±Standard Deviation of three separate experiments performed in triplicate. Data were analysed using one-way ANOVA followed by LSD in SPSS. Differences were considered statistically significant when $p<0.05$.

RESULTS AND DISCUSSION

Oxidative stress has been suggested as a causative factor for progression of diseases as well as aging. There are number of studies which link biological oxidative damage to ROS formation. Therefore the assessments of antioxidant characteristics of substances which have scavenging properties of ROS, have been a growing interest.

Antioxidants can act as either stopping the free radicals formation in the first place or stopping radical chain reactions, thus preventing biomolecular oxidation (Karadag *et al*., 2009). There are two working mechanisms of antioxidants, the one is hydrogen atom transfer and the other is electron transfer (Gulcin, 2012). The response of antioxidants to different radicals and oxidant might be different (Prior *et al*., 2005). Due to the involvement of different mechanism, there is no universal single assay to measure antioxidant capacity (Celik *et al*., 2010). Therefore different assays should be performed to evaluate antioxidant capacity.

In this study; we examined the antioxidant capacity of *Gypsophila bitlisensis* species, since its wide use in industry and medicine. Characterization of antioxidant properties of *Gypsophila bitlisensis* was determined and compared to standard antioxidants: Trolox and α-tocopherol. For those comparison three different assays were employed: Ferric cyanide (Fe^{3+}) reducing antioxidant power assay (FRAP), cupric ions reducing assay (CUPRAC) and DPPH• scavenging activity.

The FRAP assay has been widely used in the literature to determine the antioxidant capacity of the sample since it is fast, simple, robust and does not require specialized equipment. The ability of FRAP assay to measure antioxidant substance has been confirmed by the studies which showed the beneficial effect of the food on health and corresponding antioxidant capacity measured by FARP assay. For example, green tea extract has been shown to prevent collagen induced arthritis in mice (Haqqi *et al*., 1999) and FRAP assay has distinctly showed that green tea extracts increases total antioxidant capacity of heart, aorta and duodenum but not plasma in mice (Koutelidakis *et al*., 2014). Additionally, grape seed extracts has been shown to prevented colorectal cancer in mice (Velmurugan *et al*., 2010) and its antioxidant properties has been elucidated with FRAP method (Guo *et al*., 2003).

There are other assays to measure antioxidant capacity of the samples such as ABTS radical scavenging activity, Oxygen Radical Absorbance Capacity (ORAC) or the Total Radical-Trapping Antioxidant Parameter (TRAP assay). Each of these assays has advantages over the other. A comparative study has shown that although both DPPH and ABTS assay showed positive correlation with antioxidant capacity of foods, ABTS result were higher for fruits, vegetables and beverage as compared to DPPH assay (Floegel *et al*., 2011). In addition, antioxidant capacity of methanol extract of guava fruit was estimated as 31.1, 25.2, 26.1 and 21.3 determined by the ABTS, DPPH, FRAP and ORAC assay, respectively (Thaipong *et al*., 2006). On the other hand, antioxidant

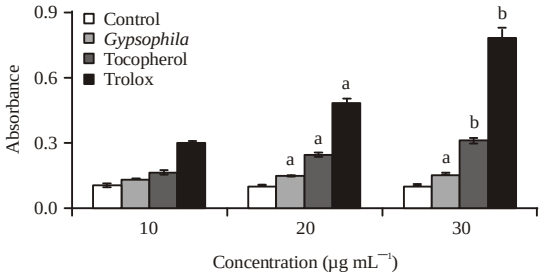

Fig. 1: Ferric cyanide (Fe^{3+}) reducing antioxidant power of *Gypsophila bitlisensis* and standards by FRAP assay

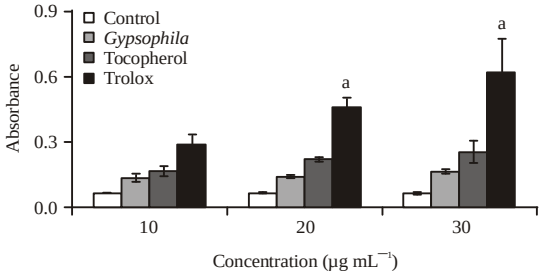

Fig. 2: Reducing power of ethanol extracts of *Gypsophila bitlisensis* and standards by CUPRAC assay

capacity of blackberry fruit were 4.19, 6.82 and 4.92 as measured by ABTS, DPPH and FRAP (Ozgen *et al*., 2006). These results indicated that antioxidant capacity may vary depending on the method, extraction solution, sample and pH used.

It has been shown that both ABTS and FRAP methods are reliable in terms of determining Total Antioxidant Capacity (TAC) of plant foods (Biskup *et al*., 2013). Although, quercetin, rutin, caffeic acid and chlorogenic acid exhibited comparable results for ABTS and FRAP assays, ferulic acid and catechin indicated higher TAC when ABTS method was used (Nilsson *et al*., 2005). The reason for higher activity might be the usage of water as a soluble in ABTS method which could increase the amount of water-soluble antioxidants in the sample (Nilsson *et al*., 2005). It was also mentioned that FRAP method is not responsive to thiol-type antioxidant measurements (Koksal and Gulcin, 2008). It this study, *Gypsophila bitlisensis* has shown slight but significant antioxidant activity at 20 µg mL^{-1} concentration and no further increase was observed at 30 µg mL^{-1} (Fig. 1). Therefore, this result showed that *Gypsophila bitlisensis* does not consist of large amount of antioxidants, as it is not highly detectable by using FRAP assay.

In order to monitor the antioxidants that may not be detectable using FRAP assay, we preformed CUPRAC assay. The principle of the method is based on the reduction of Cu^{2+}-neocuproine complex to Cu^{+}-neocuproine complex by antioxidants (Segundo *et al*., 2015). It has been first proposed by Apak *et al*. (2005) and was shown to be applicable to different biological samples including human serum

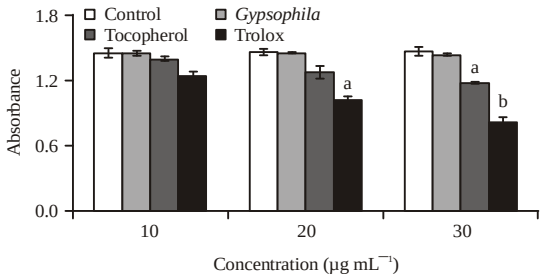

Fig. 3: Radical scavenging activity of *Gypsophila bitlisensis* and standards by DPPH assay

food and plant materials (Bener *et al*., 2010). The advantages of CUPRAC method are that it is capable of oxidizing thiol containing antioxidants, the reagents is more stable and accessible than other chromogenic reagents such as ABTS and DPPH and the reaction is carried at pH 7 which simulates physiological pH. There has been no report on evaluating the Cu reducing capacity of *Gypsophila bitlisensis* extract in the literature. This study, for the first time, showed that *Gypsophila bitlisensis* does not have a significant Cu reducing capacity as compared to trolox (Fig. 2).

The DPPH assay is also performed to monitor hydrogen atom and electron donating ability of *Gypsophila bitlisensis* extract. The mechanism is based on decolouring of purple DPPH by antioxidants which can be monitored by absorbance decrease at 517 nm. The method is rapid, easy and UV-spectrometer based. This method is powerful to show the antioxidant activity of sample as shown in previous studies. For example, using DPPH assay, researchers have shown antioxidant capacity of different plants such as fringe tree (Gulcin *et al*., 2006), vegetables (cauliflower) (Koksal and Gulcin, 2008), fruits (guava) (Thaipong *et al*., 2006) leaves (*Melissa officinalis*) (Koksal *et al*., 2011). In this study, *Gypsophila bitlisensis* extract did not show a significant reduction in DPPH scavenging activity (Fig. 3).

The phenolic compounds such as lignin, flavonoid or tannin can be considered as a large group of secondary metabolites of plants (Blokhina and Fagerstedt, 2010). Polyphenol structures have ideal properties for antioxidant system and some polyphenols can show more antioxidant activity *in vitro* than vitamin E or C. Furthermore, different mechanisms are proposed (Rice-Evans *et al*., 1997). The food phenolics exhibit important structural diversity and can be categorized as many compounds class. The total phenolic content in many plants have been determined such as sumac (Bursal and Koksal, 2011), orange (Rapisarda *et al*., 1999), cherry stem (Bursal *et al*., 2013) and cauliflower (Koksal and Gulcin, 2008). The method used for determination total phenolic content in *Gypsophila bitlisensis* was already explained in the study of Koksal *et al*. (2011). In this study, we have found that total phenolic amount of *Gypsophila bitlisensis* was 8.75 µg GAE.

Gypsophila saponin has been found to have blood cholesterol lowering effect in rats, due to the possible

interference with Fe metabolism (Southon *et al*., 1988). Therefore it would be useful to determine Fe^{3+} reducing capacity of *Gypsophila bitlisensis* which may explain antioxidant characteristics of it, if has any.

In summary oxidative stress theory has been widely accepted to explain cellular damage in diseases and aging. Therefore discoveries of plant rich in antioxidant are important to treat and prevent the oxidation of cellular components. In this study we have shown that *Gypsophila bitlisensis* does not have considerable antioxidant capacity as measured by three different assays: The FRAP, CUPRAC and DPPH scavenging activity. Further studies should be performed to explore herbs and plants that can be used in medicine.

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