Evaluation of Antioxidant Activity of Various Herbal Folk Medicines

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Abstract

The leaves of some plants are flamed and used to dress injured skin, to stimulate healing and to ward off infection. This study aims to determine antioxidant activities of Plantago lanceolata L., Plantago major L. ssp. major, Rubus hirtus Waldst&Kit., Sambucus ebulus L., Morus alba L. and Hedera helix L., which grown in Düze and its surroundings and are used for anti-inflammatory purposes, and especially for wound-healing. The total phenolic content of these plants was also evaluated. In order to determine the antioxidant compounds and antioxidant capacity of these plants, different methods were studied. Resulting from our study, while P. lanceolata, R. hirtus, S. ebulus and P. major ssp. major had similarities in terms of phenolic compounds content, M. alba and H. helix were found to have relatively low content of phenolic compounds. In general, Plantago species and R. hirtus showed high antioxidant activities.

Keywords: Antioxidant activity; Plantago major ssp. major; Plantago lanceolata; Rubus hirtus; Hedera helix; Morus alba; Sambucus ebulus

Introduction

It has been established that oxidative stress is among the major causative factors in induction of ageing and immunosuppression, and many chronic and degenerative diseases including atherosclerosis, ischemic heart diseases, diabetes mellitus, cancer, neurodegenerative diseases and others [1]. Natural antioxidants can protect the human body from free radicals and retard the progress of many chronic diseases as well as retarding the lipid oxidative rancidity in foods [2]. Recent studies have investigated the potential of plant products as antioxidants against various diseases induced by free radicals [3].

There is an increasing interest in natural antioxidants, e.g., polyphenols, present in medicinal and dietary plants, which might help preventing oxidative damages [4]. It has been determined that the antioxidant effect of plant products is mainly attributed to phenolic compounds such as flavonoids and phenolic acids [5].

Plantago lanceolata and Plantago major are species of Plantago genus including 275 species throughout the world. Their leaves are used as astringent, anti-inflammatory, expectorant, topical anodyne, antibacterial, styptic, hemoptysis, hematuria, sore throats, coughs, diarrhea, dysentery, hemorrhoids, cervices, rectal fissures, insect bites, snake bites, cuts, bruises and abscess. These Plantago sp. consist several compounds, including iridoid glycosides (aucubin, catapal, gardsoside, geniposidic acid, mayorside, melittoside), terpenoids (loliolid, usorolic acid, oleanolic acid), caffeic acid derivatives (caffeic acid, chlorogenic acid, plantamajoside R, acetoside R), polysaccharides (plantaglucide, glucomanon, PMII, PMI), alkoloids (indicain, plantagonin), polyholozide, flavonoids and flavon glucosides (luteolin-7-glucoside, hispidulin-7-glucuronoide, apigenin, balcalein, scutallarin, plantagonin) [6].

Rubus hirtus is commonly called as blackberry belonging to family Rosaceae. It is present and widespread in the Turkey. The leaves of R. hirtus are used for abscess and to stop bleeding in cuts [7] and contains quercetin, kaempferol, caffeic acid and chlorogenic acid); phenolic acids, tannins, amino acids, sugars, pectins, carboxylic acids, anthocyanins, catechins, vitamin C and saturated or unsaturated fatty acids [8-11].

Sambucus ebulus belong to family Caprifoliaceae. S. ebulus grows mainly in the north of Turkey. Its leaves used for the treatment of inflammatory reactions, such as hemorrhoid, bites and sore-throat. The phytochemical compounds of S. ebulus are flavonoids, steroids, tannins, glycosides, cardiac glycosides, caffeic acid derivatives, ebultins, ebulin 1 and volatile substances [12-18]. Traces of a cyanogenic glucoside, sambunigrin and the triterpenes alpha- and beta-amyrin were isolated from leaves, roots and fruits.

Morus alba is known as white mulberry belonging to Moraceae family. Its leaves are used as a blood pressure depressant. Chemical constituents of Morus alba leaves are polyfilavanals, glycoside, isoquercitrin, astragalin, scopolin, skimmia, roseoside II and benzyl D-glucopyranoside [19].

Hedera helix, ivy, is a species of Hedera genus in the family Araliaceae, native to most of Europe and western Asia. Hedera helix leaves are used for wound healing, gout, rheumatism and externally against parasites, and to treat burns. The leaves of Hedera helix consist many aktive compounds such as hederagenin, oleanolic acid and bayogenin, o-hederin, hederagenin-3-O-D-glucoside, hederasperonin C (hederacoidise C) with other hederasapornins (B, D, E, F, G, H and I), flavonoids such as quercetin, kaempferol, isoquercitrin and astragalin, phytosterol as stigmasterol, sitosterol, cholesterol, campesterol, ß-siphersterol, coumarin glycoside scopolin and the polyacetylenes falcarnine, falcariol and 11, 12-dihydrofalcariol [20].

Since ancient times, herbs have been routinely used to treat wounds, and in many cultures their use in traditional medicine has persisted to the present [21]. In Düze, plants are often used for wound healing, stomach soothing, curing intestinal disorders and coughs, and for healing burns. The ways of using plants are generally the same: fresh

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leaves of plants, infusion, decoction, poultice and expressed juice from fresh plants.

The plants in this study were selected from among those used especially for wound-healing in Düzce and its surroundings. The study was designed to examine antioxidant characteristics of Plantago lanceolata L., Plantago major L. ssp. major, Rubus hirtus Waldst & Kt., Sambucus ebulus L., Morus alba L. and Hedera helix L. which used as anti-inflammatory. In addition, through assessing total phenolic contents of the plants aimed to investigate whether there is a relationship between phenolic content and antioxidant activity.

Materials and Methods

Chemicals

1,1-diphenyl-2-picryl-hydrazyl (DPPH) (Aldrich), N, N-dimethyl-p-phenylenediamine dihydrochloride (DMDP) (Fluka), ferric chloride (Aldrich), amonium acetate (Fluka), 2, 2’-azinobis (3-ethylbenzothiazoline-6-sulphonic acid diammonium salt) (ABTS) (Sigma), linoleic acid (Fluka), Tween 20 (Sigma), ethanol (Merck), methanol (Merck), ammonion thioaciane (Sigma & Aldrich), nicotinamide adenine dinucleotide (NADH) (Sigma), nitrogen blue tetrazolium chloride (NBT) (Sigma), phenazine methosulphate (PMS) (Sigma), 30% hydrogen peroxide (Fluka), Folin-Ciocalteu reagent (Sigma & Aldrich), sodium carbonate (Na2CO3) (Sigma & Aldrich), sodium hydroxide (NaOH) (Merck), copper(II)sulfate (CuSO4) (Sigma & Aldrich), Total Antioxidant Response kit (Abbott).

Plant material and extraction

P. lanceolata, P. major ssp. major, R. hirtus, S. ebulus, M. alba and H. helix were collected from Düzce and its surroundings. Plants were dried in the shadow for extraction. Plants were collected in June. The purposes of use of plants in this region are given at the Table 1.

Folin-Ciocalteu method for total phenolic content

The amount of total soluble phenolics was determined according to the Folin-Ciocalteu method [23], modified for use by a plate reader. Folin Ciocalteu's phenol reagent was diluted to a volume ratio of 1:3 with 96% deionised water before used. Lowry A solution was prepared from sodium carbonate so that the strength (w/v) of Na2CO3 in 0.1 M NaOH solution was 2%. Lowry B solution was prepared from copper(II) sulphate so that the strength (w/v) of CuSO4 in 1% sodium potassium tartrate (NaKC4H4O6) solution was 0.5%. Lowry C solution was prepared by freshly mixing 50 ml Lowry A with 1 ml Lowry B. To 100 µl of herb extract, 125 µl Lowry C was added. The mixture was mixed on a microplate shaker at 250 rpm. Then, 12.5 µl Folin reagent was added and 30 min was allowed for stabilization and to turn blue in colour. The absorbance against a reagent blank was measured at 750 nm.

CUPRAC assay

Cuprasc is based on reduction of Cu(II) to Cu(I) by antioxidants (reductant) which are present in the sample [24]. We modified this assay to work with 96-well plates. CuCl2 solution, 1.0×10-2 M, was prepared by dissolving 0.17 g CuCl2.2H2O in water, and diluting to 100 ml. Ammonium acetate buffer at pH 7.0, 1.0 M, was prepared by dissolving 19.27 g NH4Ac in water and diluting to 250 ml. Neocuproine (Nc) solution, 7.5×10-3 M, was prepared by dissolving 0.039 g Nc in 96% ethanolic. 50 µl CuCl2 solution (10-2 M), 50 µl neocuproine alcoholic solution (7.5×10-3 M) and 50 µl NH4Ac buffer solution, 27.5 µl sample and 27.5 µl water were added to wells. The mixture is then mixed well and incubated the microwell strips at room temperature (18° to 25°C) for about 30 min in dark. After that, absorbance was measured against a reagent blank at 450 nm.

DPPH assay

The free radical scavenging activity of these plants was measured by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) method, modified for working with 96 well plate. With this method it was possible to determine the antiradical power of an antioxidant by measuring the decrease in the absorbance of DPPH at 517 nm. As a result of the color changing from purple to yellow the absorbance is decreased when the DPPH radical is scavenged by an antioxidant through donation of hydrogen to form a stable DPPH-H molecule [25]. This method was applied to ethanolic, methanolic and aqueous extracts of plants (1000-500-250-125 µg/ml). First, 0.1 mM solution of DPPH in MeOH was prepared. Then, 50 µl of this solution was added to 150 µl of extracts of plants. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm in an ELISA reader. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity [26]. The DPPH scavenging effect calculated using following equation:

\[
= \frac{[\text{Abscontrol}-\text{Abssample}]}{\text{Abscontrol}} \times 100
\]

| Plant name          | Part used | Use                                                                 |
|---------------------|-----------|----------------------------------------------------------------------|
| Plantago lanceolata L. (Plantaginaceae) | Leaf | For stomach ache, intestinal disorders, internal disorders, maturation of an abscess, shortness of breath, diabetes, wound healing and to treat burns. |
| Plantago major L. subsp. major (Plantaginaceae) | Leaf | For the maturation of an abscess, hemorrhoids, kidney cancer, wound healing, urinary infection, bronchitis, cough, diarrhea, stomach bleeding, ulcer, stomach ache and as a panacea. |
| Rubus hirtus Waldst & Kt. (Rosaceae) | Leaf | For cuts, healing wound, anemia and to treat stomach ache. |
| Sambucus ebulus L. (Caprifoliaceae) | Leaf | For bee stings, pain, vaginal pain after birth, wound healing, malaria and to treat swellings of breasts in cattle, rheumatic pain. |
| Morus alba L. (Moraceae) | Leaf | For wound healing. |
| Hedera helix L. (Araliaceae) | Leaf | For wound healing and to treat burns. |

Table 1: Folk Remedies in Düzce.
DMPD assay

DMPD assay modified for use by a plate reader. DMPD (100 mM) was prepared by dissolving 209 mg of DMPD in 10 ml of deionised water. This solution (1 ml) was added to 100 ml of 0.1 M acetate buffer, pH 5.25, and the coloured radication cation (DMPD+) was obtained by adding 0.2 ml of a 0.05 M solution of ferric chloride (the final concentration was 0.01 mM). This solution (225 µl) was transferred directly to the microwell and its absorbance at 505 nm was measured (A0). Samples (15 µl) were added to all wells. Then added 210 µl of DMPD+ to all samples and, stirred and left to stand for 10 min. After this period, a decrease in absorbance was measured (A1). The DMPD solution was used as control [27].

The DMPD+ scavenging effect calculated using following equation:

\[ \frac{[A_0 - A_1/A_0] \times 100}{100} \]

ABTS assay

ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation (ABTS+), was produced by reacting ABTS stock solution with 2.45 mM potassium persulphate (final concentration) and allowing the mixture to stand in the dark, at room temperature for 12-16 h before use. The kept solution was diluted with 96% ethanolic at a ratio of 1:30. Respectively 10 µl sample, 200 µl ABTS, was added to wells. 210 µl of ABTS. solution was used as control (A0). Then, the change in absorbance during 5 min was recorded and measured at 734 nm by an ELISA reader [28].

The ABTS scavenging effect calculated using following equation:

\[ \frac{[A_0 - A_1/A_0] \times 100}{100} \]

Ferric thiocyanate assay

The reaction mixture consisted of 0.28 g linoleic acid, 0.28 g of Tween 20 and 50 ml of phosphate buffer (0.05 M, pH 7.4). To 1.25 ml of the above linoleic acid emulsion, 0.1 ml of test sample and 1.25 ml of phosphate buffer (0.2 M, pH 7.0) were added and incubated at 40°C for 24 h. The mixture prepared, as above, without the test sample, was the control. After 24 h, 0.1 ml of the mixture was taken and mixed with 4.7 ml of 75% ethanolic, 0.1 ml of 30% ammonium thiocyanate and 0.1 ml of 20 mM ferrous chloride in 3.5% HCl and allowed to stand at room temperature. Precisely 3 min after the addition of ferrous chloride to the reaction mixture, the absorbance at 500 nm was measured [29].

Scavenging of superoxide

The reaction mixture, which contained 50 µl of sample, 50 µl of NADH (936 µM) in phosphate buffer (100 mM, pH 7.4), 50 µl of NBT (300 µM) in phosphate buffer and 50 µl of PMS in phosphate buffer was incubated at room temperature for 5 min. The absorbance was read at 560 nm by an ELISA reader, against blank samples (A0). The reaction mixture, without the extract was used as control (A1) [30].

The superoxide scavenging effect calculated using following equation:

\[ \frac{[A_0 - A_1/A_0] \times 100}{100} \]

Scavenging of hydrogen peroxide

A solution (43 mM) of hydrogen peroxide was prepared in phosphate buffer (0.1 M pH 7.4). 400 µl of sample was mixed with 960 µl phosphate buffer. Then, 240 µl of hydrogen peroxide solution (43 mM) was added to the mixture. Absorbance was read at 230 nm by a spectrophotometer against blank samples (A0). The reaction mixture, without the extracts was used as control (A0) [31].

The hydrogen peroxide scavenging effect calculated using following equation:

\[ \frac{[A_0 - A_1/A_0] \times 100}{100} \]

Total Antioxidant Response (TAR)

To 5 µl herbal extract, 200 µl R1 (o-dianisidine) was added. The first absorbance was taken before mixing R1 and R2 (as sample blank). After 4.5 min, 10 µl of R2 (H2O2) was added to the mixture and the last absorbance was taken when the reaction trace drew a plateau line (about 3-4 min after the mixing). Measurements were taken by an ELISA reader at 444 nm [32].

Folin-Ciocalteu method for total phenolic content

The total phenolic contents of the plants were determined from the regression equation of gallic acid calibration curve (y=0.4685 ln(x)-1.131) (Figure 1). The concentrations of total phenolic content of extracts of plants are shown in Table 2. The highest amount was found in the P. lanceolata, followed by R. hirtus, S. ebulus, P. major ssp. major, H. helix and M. alba, respectively.

CUPRAC assay

Cupric is one of the assays to determine the total antioxidant activity, which uses cupper II as an oxidant. This method is based on the reduction of Cu(II) to Cu(I) by antioxidants (reductant), which present in the sample. This method measures the capacity of hydrophilic and lipophilic antioxidants. On the first trial, standards gave good results and the same results were obtained from extracts. Trolox (a water-soluble tocopherol analogue), gallic acid and vitamin C were used as standard solutions. We observed a good correlation between the concentration and percentage inhibition(y=0.0032 ln (x)-0.0379, R²=0.9985 for trolox, y=0.4685 ln (x)-1.131, R²=0.9893 for gallic acid, y=0.0024 ln (x)-0.2221, R²=0.9695 for vitamin C). According to the results of the cupper reducing antioxidant capacity (CUPRAC) assay, R. hirtus and P lanceolata showed the highest antioxidant activity, M. alba showed the lowest activity in aqueous and methanolic extracts (Figure 1a and 1b). In ethanolic extracts, while P lanceolata exhibited the

| Plant | Total phenolic content (µg/mL) |
|-------|-------------------------------|
| Plantago lanceolata L. | 146.368 |
| Rubus hirtus Waldst&Kit | 144.814 |
| Sambucus ebulus L. | 135.542 |
| Plantago major L. ssp. major | 107.407 |
| Hedera helix L. | 39.555 |
| Morus alba L. | 13.721 |

Table 2: Total phenolic content of plants.
highest antioxidant activity, *M. alba* again showed the lowest activity (Figure 1c).

**DPPH assay**

DPPH free radical scavenging is one of the generally accepted mechanisms against lipid oxidation. Difference between DPPH free radical binding method and the other method is the short run time allowing rapid determination of the radical scavenging. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability. The antioxidant activity of plant extracts were calculated according to the percentage inhibition in DPPH assay. Gallic acid was used as the reference standard \( y = 0.9221 \ln (x) + 0.039, R^2 = 0.9925 \). The DPPH free radical scavenging activity of the plant extracts are shown in Figure 2a-2c. While *R. hirtus* had the highest activity in aqueous and methanolic extracts, *P. lanceolata* showed the highest DPPH free radical scavenging activity in ethanolic extracts. *M. alba* showed the lowest DPPH free radical scavenging activity in aqueous, methanolic and ethanolic extracts.

**DMPD assay**

DMPD method is suitable for fast and sensitive measurement of antioxidant activity of hydrophilic compounds. In our study, although we observed high correlations in DMPD radical and Trolox standard studies, we couldn't reach the same consistency in all plant extracts. However, similar results to those in other methods were observed in methanolic extracts gave the most uniform results. Different
concentration of DMPD was carried out \((y=0.1339 \ln(x)-0.083, R^2=0.9875)\) and trolox was used as standard \((y=17.771 \ln(x)-63.376, R^2=0.9901)\). The antioxidant activity of plant extracts were calculated according to the percentage inhibition in DMPD assay. According to our results \(R. hirtus\) gave the highest antioxidant activity in methanolic extracts, \(P. major\) ssp. \(major\) gave the highest antioxidant activity in aqueous extracts and \(P. lanceolata\) gave the highest antioxidant activity in ethanolic extracts (Figure 3a and 3c). While \(S. ebulus\) had the lowest antioxidant capacity in ethanolic extracts, \(M. alba\) showed the lowest antioxidant capacity in methanolic and aqueous extracts (Figure 3b).

**ABTS assay**

ABTS method is commonly used to assess radical scavenging or antioxidant activity of the sample on the inhibition of ABTS radical (ABTS), which is a synthetic compound and is produced by oxidation of ABTS. ABTS assay is based on the decolourization of blue-green ABTS radical cation, which is reduced by antioxidant compounds, plant, and fruit extracts. Trolox, which was observed to have a good correlation between the concentration and percentage inhibition \((y=-0.0001 x^2+0.2374x-3.2115, R^2=0.9953)\), was used as standard. According to our results, \(P. lanceolata\), \(P. major\) ssp. \(major\), \(R. hirtus\), and \(S. ebulus\) gave almost the same antioxidant activity, \(M. alba\) showed the lowest activity in aqueous extracts (Figure 4a). Outside the small differences, we observed roughly similar results in methanolic and ethanolic extracts (Figure 4b and 4c).

**Ferric thiocyanate assay**

In our study, inhibition of lipid peroxidation capacity of aqueous...
Ferric Thiocyanate Method

Figure 5: Total antioxidant activities of aqueous extracts of plants was determined by ferric thiocyanate method in linoleic acid emulsion.

plants were investigated by ferric thiocyanate assay, which is based on the oxidation of fats and oxidizing Fe$^{2+}$ to Fe$^{3+}$ by peroxides and Fe$^{3+}$ ions which occur in this reaction, making it rather complex with SCN. Decrease in the absorbance indicates an increase in the free radical scavenging activity. In this assay, gallic acid was used as standard ($y=-0.224 \ln (x)+1.6333, R^2=0.9673$). The results showed that $P. lanceolata$ had the highest antioxidant activity followed by $P. major$, $R. hirtus$, $H. helix$, and $S. ebulus$. The present work demonstrated that the aqueous extract of $M. alba$ leaves did not show remarkable activity (Figure 5).

Scavenging of superoxide

Superoxide is accepted to be a very harmful radical that can be converted into more reactive species, such as hydroxyl radical or peroxynitrite, contributing to tissue damages and various diseases. The superoxide radical assay of samples was measured according to the method reported by Robak and Gryglewski [30]. The scavenging activity of superoxide radical of plant extracts was measured and the percentage inhibition was calculated. Different concentration of vitamin C and gallic acid used as standard ($y=-6E-05 x^2+0.109x+25.947$ for vitamin C, $R^2= 0.9994$ and $y=12.485 \ln (x)+2.186, R^2=0.9848$ for gallic acid). According to the data $R. hirtus$ gave the highest antioxidant activity and $M. alba$ showed the lowest activity in aqueous and ethanolic extracts (Figure 6a and 6c). In methanolic extracts, while $R. hirtus$ exhibited the highest antioxidant activity, $H. helix$ showed the lowest activity (Figure 6b).

Scavenging of hydrogen peroxide

Hydrogen peroxide ($H_2O_2$) is a weak oxidizing agent but it can cross cell membranes rapidly. Once inside the cell, $H_2O_2$ can react with Fe$^{2+}$ ions to generate the hydroxyl radical and this may be the origin of many of its toxic effects [33]. $H_2O_2$ is relatively stable in the absence of reducing compounds. Scavenging of $H_2O_2$ by plant extracts may be attributed to their electron donating abilities [34]. Hydrogen peroxide scavenging activity of the plant extracts is presented in Figure 7; In this method, we used only one concentration (50 μg/mL) of all extracts. In aqueous extracts, $M. alba$ showed the highest activity and $P. lanceolata$ showed the lowest activity in methanolic and ethanolic extracts.

Total antioxidant response (TAR)

In the last method, to measure total antioxidant response, the
hydroxyl radical is produced by the Fenton reaction and oxidize colourless substrate O-dianisidine to the dianisyl radical, which is stabilized. Therefore, it is important to determine the total amount of phenolics to determination of antioxidant capacity of plants. In our study, we found higher phenolic content in P. lanceolata, R. hirtus, S. ebulus and P. major ssp. major than those in H. helix and M. alba.

Phenolic compounds are herbal substances whose chemical structures may range from quite simple compounds to highly polymerized substances. The capacity of phenolics to scavenge free radicals may be due to many phenolic hydroxyl groups they possess. Phenols play an important role in antioxidant activity, because they transfer hydrogen to radicals and produce phenoxide radical, which is stabilized.

There are few studies carried out in Turkey showing high antioxidant activities for these plants, which are astringent, anti-toxic, antimicrobial, anti-inflammatory, expectorant and diuretic. Some of the compounds in Plantago sp. such as ursoic acid, plantamajoside R, acetoside R and plantagonin have been shown to have both an antioxidant and anti-inflammatory effect [6]. Numerous studies have shown that Plantago species had a high phenolic content and antioxidant activity [39-48]. There are few studies carried out in Turkey showing high antioxidant activity for P. lanceolata [40-50] and P. major [51,52]. In our study, we also observed high content of phenolic compounds and antioxidant activity for P. major ssp. major and P. lanceolata in some of the methods.

In several studies, very high antioxidant activities were observed in different types of Rubus (Rubus ursicifolius Poir., Rubus parviflorus Nutt.) [53-57]. Although anti-inflammatory and antinociceptive effects of R. hirtus have shown in previous studies [58,59], only one study has specifically investigated the antioxidant activity of its fruit [60]. As far as we know, this is the first study showing antioxidant activity in the leaves of R. hirtus. According to our results, its antioxidant activities were mostly higher than other plants studied and we think that these high antioxidant activities of R. hirtus may contribute to its anti-inflammatory effects.

S. ebulus has shown high antioxidant activity in generally and a positive correlation was observed between antioxidant activity and phenolic content [61,62]. According to our results, although the phenolic content of S. ebulus was close to P. lanceolata and R. hirtus, their antioxidant properties were not similar. However, high levels of thiocyanate found in S. ebulus indicate its ability to inhibit lipid peroxidation. According to our knowledge, this is the first study, which has investigated the antioxidant activity of S. ebulus in Turkey.

It has been shown that alpha hederin and hedera saponin C component of H. helix had a strong total antioxidant activity and inhibits lipid peroxidation [63-67]. However, H. helix showed lower total phenolic content and lower antioxidant activity when compared with the other species.

Discussion

The process of inflammation may be initiated by an infection or cell damage that stimulates the secretion of interleukins or tumour necrosis factor by macrophages. Activated neutrophils produce large amounts of ROS, thus causing the production of toxic reactive species to increase. Neutrophils also release myeloperoxidase (MPO). Myeloperoxidase (MPO) catalyzes the reaction of hydrogen peroxide with chloride ion to produce hypochlorous anion. Also reaction of hypochlorous anion with hydrogen peroxide yields singlet oxygen, which is a reactive species. The interaction of superoxide radical with hydrogen peroxide and iron is causing the formation of hydroxyl radical, which is highly reactive, and the reaction of superoxide with nitric oxide is resulting in the formation of peroxynitrite. These reactive species kill pathogens by halogenations or by protein oxidation and lipid peroxidation. If the balance between oxidants and antioxidants shifts in the direction of oxidants during the inflammation, healthy cells can get damaged [35,36].

Plant-derived antioxidants such as tannins, lignans, stilbenes, coumarins, quinones, xanthones, phenolic acids, flavones, flavonols, catechins, anthocyanins and proanthocyanins could delay or prevent the onset of degenerative diseases because of their redox properties, which allow them to act as hydrogen donors, reducing the agents, hydroxyl radicals (OH.) or superoxide radical (O2•-) scavengers [37,38].

Phenolic compounds are herbal substances whose chemical structures may range from quite simple compounds to highly polymerized substances. The capacity of phenolics to scavenge free radicals may be due to many phenolic hydroxyl groups they possess. Phenols play an important role in antioxidant activity, because they transfer hydrogen to radicals and produce phenoxide radical, which is stabilized. Therefore, it is important to determine the total amount of phenolics to determination of antioxidant capacity of plants. In our study, we found higher phenolic content in P. lanceolata, R. hirtus, S. ebulus and P. major ssp. major than those in H. helix and M. alba.

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It has been shown that alpha hederin and hedera saponin C component of H. helix had a strong total antioxidant activity and inhibits lipid peroxidation [63-67]. However, H. helix showed lower total phenolic content and lower antioxidant activity when compared with the other species.
to other plants in our study. Similarly, in previous studies, leaves [68-70], fruit [71-73] and bark [74-76] extracts of *M. alba* showed high antioxidant activity. In our study, lowest phenolic compounds and antioxidant activity was observed in *M. alba*.

It is thought to be due to the different time and collection zone of study materials. In a previous study, Sivaci and Sökmen showed *M. alba* antioxidant activity was observed in *M. alba*. In our study, lowest phenolic compounds and antioxidant activities, it could be useful for the interpretation of such results. Additionally, if we had looked for their anti-inflammatory activities, it could be useful for the interpretation of such results. Because seasonal changes affect the contents of plants too, it is difficult to compare the activities of different plants. Observation of different antioxidant activities of some plant species which have been demonstrated their anti-inflammatory effect in the literature and used by people for this purpose can be interpreted in different ways. Because of their use for different purposes other than anti-inflammatory effects, and knowing their major effects makes it easy to comment on the results. For example, *S. ebulus* has been mostly used as a pain killer, as an anti-inflammatior and has showed a relatively lower antioxidant activity in our study. In conclusion, if we had looked for their anti-inflammatory activities, it could be useful for the interpretation of such results.

Although it is not our main goal, we modified Folin, Ciprac, and DPPH methods to work with 96 well plates. This would be useful for those who will work with these methods.

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