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Comparative Study of Antioxidant Properties and Total Phenolic Content of the Extracts of *Humulus lupulus* L. and Quantification of Bioactive Components by LC−MS/MS and GC−MS

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Supporting Information

ABSTRACT: In this research, antioxidant activities of various extracts obtained from *Humulus lupulus* L. were compared by DPPH, ABTS, FRAP, and CUPRAC assays. The amount of total phenolic components determined by the Folin-Ciocalteu reagent was found to be highest for 25% aqueous ethanol (9079 ± 187.83 mg Ferulic acid equivalent/100 g extract) and methanol-1 (directly) (8343 ± 158.39 mg Ferulic acid equivalent/100 g extract) extracts. The *n*-hexane extract of *H. lupulus* exhibited the greatest with DPPH (14.95 ± 0.03 μg Trolox equivalent/g sample). The highest phenolic content in the ethanolic extract could be the major contributor to its highest CUPRAC activity (3.15 ± 0.44 mmol Trolox equivalent/g sample). Methanol-2 (*n*-hexane, acetone, and methanol) and methanol-3 (*n*-hexane, dichloromethane, ethylacetate, and methanol) extracts, respectively, exhibited the most potent ABTS (7.35 ± 0.03 mM Trolox equivalent) and FRAP (1.56 ± 0.35 mmol Fe^{2+}/g sample) activities. Some of the components from the crude extracts were determined by LC−MS/MS and GC−MS analyses. Comparative screening of antioxidant activities of *H. lupulus* extracts and quantification of some major components by LC−MS/MS, qualitatively analysis of the reported ones which were optimal under negative ion SIM mode and coinjection, are going to be valuable for food and health applications.

KEYWORDS: *Humulus lupulus, hop cones, total phenolic content, DPPH, CUPRAC, ABTS, FRAP, GC−MS, LC−MS/MS, LC−MS (SIM mode), bioactive components*

INTRODUCTION

Phenolic compounds are ordinarily found in both edible and nonedible plants, and they have been reported to have many biological effects, including antioxidant activity. Crude extracts of fruits, herbs, vegetables, cereals, and other plant materials rich in phenolics are increasingly of interest in the food industry because they decelerate oxidative degradation of lipids and therefore improve the quality and nutritional value of food. The importance of the antioxidant constituents of plant materials in the maintenance of health and preservation from coronary heart disease and cancer is also raising interest among scientists, food manufacturers, and consumers as the tendency of the future is moving toward functional food with specific health effects.[1] Flavonoids and other phenolics have been suggested to play a protective role in the improvement of cancer and heart disease.[2]

Hop (*Humulus lupulus* L.) is a dioecious perennial plant belonging to the Cannabaceae. The plant of *H. lupulus* is well-known throughout the world as the raw material in the brewing industry. The female inflorescences (hop cones, strobiles, or "hops"), rich in polyphenolic components and acyl phloroglucides, are widely used to protect beer and to give it a characteristic aroma and flavor. In addition hop cones have long been used for medicinal targets. In particular, hop preparations were mainly suggested for the treatment of sleeping disorders, as a mild sedative, and for the activation of gastric function as bitter stomachic.[3] In line with the growing interest in the health benefits of plants used in traditional medicine, *H. lupulus* has received considerable interest by researchers, and as a result, an important number of articles have been published. In the second half of the 20th century, several phytochemical studies were undertaken to investigate the constitution of hop cones and other parts of the plant, leading to the isolation and identification of pharmacologically relevant compounds such as flavanones, chalcones, and phloroglucinol derivatives.[3] Dried hop flowers (hops) have attracted a great deal of attention as a source of small molecules such as humulones, lupulones, isohumulones, and xanthohumol with the prospective for beneficial effects on human health. It is known that these naturally occurring molecules show antibacterial, antioxidant, antiinflammatory, and anticancer activities.[3−6]

In recent years, researchers have been working to define the bioactive ingredients in hops and to illuminate the underlying molecular mechanisms by which they perform their activities. Much attention has gone to the polyphenolic content of hops, and specific compounds, such as xanthohumol and 8-prenylnaringenin, have been described as multipotent bioactive

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constituents.[7−9] Furthermore, increasing evidence shows that the so-called hop bitter acids, which represent up to 30% of the total lupulin content of hops, display interesting effects on human health.[10] In the early nineteenth century, extraction of hops was first attempted in water and ethanol,[11] but other methods have been also reported, such as the use of steam or carbon disulfide.[12] The production of hop extracts has been developed in the last century, when the chemical structure and reactivity of the resin compounds were elucidated. Due to their lipophilic nature, a broad range of efficient solvents, including alcohols, chloroform, acetone, and *n*-hexane, have been used to dissolve the resin constituents.[3]

Various health-promoting effects of plant compounds can be attributed to their main antioxidant activities: they neutralize cell damage caused by reactive oxygen species (ROS) and reactive nitrogen species such as free radicals, singlet oxygen, and hydroperoxides. Cell damage caused by free radicals seems to be a major contributor to aging and degenerative diseases of aging such as cancer, cardiovascular disease, immune system decline, diabetes mellitus, inflammation, brain dysfunction, and stress, among others. Phytochemicals may assist the body's own defense enzymes, such as superoxide dismutase and glutathione peroxidase, to scavenge or quench free radicals to protect the body against hazardous effects.[10] Several assays have been frequently used to estimate antioxidant capacities in plant extracts including DPPH, ABTS, FRAP, and CUPRAC assays.[13−18] These techniques have indicated different results among plants tested and across laboratories.[19]

In this research, various solvents were used to extract the hydrophilic antioxidants present in this plant. Therefore, the aim of this study was to determine the total phenolic content and to characterize the antioxidant activities of *H. lupulus*, currently used in the beer industry for aroma and food flavoring applications, in order to determine their potential in nutraceutical formulations, using DPPH, ABTS, FRAP, and CUPRAC assays, and to determine the components of crude extracts by LC−MS/MS and GC−MS analyses.

MATERIALS AND METHODS

Chemicals. ABTS, DPPH, TPTZ, ferric chloride, Folin-Ciocalteu's phenol reagent, Trolox, quercetin, neocuproine, and ferulic acid (99%) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). All of the solvents were of analytical grade and obtained from Merck.

Plant Material. The cones of *H. lupulus* weighing 1.0 kg were collected from Balıkesir (wayside; altitude, 55 m) and Darıca village district of Manyas in western of Turkey in the month of September 2010 and voucher specimens collected were identified, processed, and deposited in the Herbarium of Uludağ University Biology Department of Bursa in Turkey (voucher no: 32808). After harvest, the plant material weighing 780 g was air-dried under shade at room temperature for 2 months. Plant material was removed from moisture before pulverizing in the incubator at 70 °C and then stored in the fridge (4 °C).

Extraction of Plant Material. Different solvents, e.g., (*n*-hexane, dichloromethane, ethylacetate, acetone, methanol, and 25% aqueous ethanol) were used to determine the effectiveness of solvent type on the extraction of phenolics from *H. lupulus.* The pulverized plant material was divided into four parts for extraction. First, the Soxhlet-extraction of the plant material (100 g) was carried out sequentially, using *n*-hexane (a), dichloromethane (b), ethylacetate (c), and methanol (d) (methanol-3) (each 1 L). Then, hop cones (100 g) of *H. lupulus* were extracted directly with methanol (e) (methanol-1) (1 L). Next, plant material (100 g) was extracted with *n*-hexane, acetone (f), and methanol (g) (methanol-2) (1 L). In addition, plant material (100 g) was extracted with 25% aqueous ethanol (h) (1 L). Finally, filtrates were concentrated in vacuo at ambient temperature and stored at the +4 °C.

Thin-Layer Chromatography. Total plant extracts were examined by thin-layer chromatography on silica gel plates (Merck). Organic acids in *n*-hexane extract were analyzed in *n*-hexane-ethylacetate (4:1, v/v) and glasial acetic acid (three drops). The other extracts were sprayed after migration in *n*-hexane/ethylacetate and dichloromethane/methanol (4:1, v/v) by DPPH solution (8 mg/100 mL) in methanol. It was allowed to develop for 30 min. The color changes (purple on white) were observed at 254 nm. Thus, active extracts were determined by the DPPH qualitative assay.[22]

Determination of Total Phenolic Contents. The amount of total phenolics was determined according to the method of Velioglu et al. which used the Folin-Ciocalteu reagent. Extract was prepared at a concentration of 1 mg/mL. Extract (100 μL) was transferred into a test tube, and 0.75 mL of Folin-Ciocalteu reagent (previously diluted 10-fold with deionized water) was added and mixed. The mixture was allowed to stand at room temperature for 5 min. Sodium carbonate (0.75 mL of 6% w/v) was added to the mixture and then mixed gently. After the mixture was allowed to stand at room temperature for 90 min, the absorbance was read at 725 nm using a UV−vis spectrophotometer. The standard calibration (0.01−0.05 mg/mL) curve was plotted using ferulic acid (r = 0.98). The total phenolic content was expressed as ferulic acid equivalents in milligram per 100 g of vegetable extract. All determinations were performed in triplicate.[23]

Evaluation of Antioxidant Activity. *ABTS Radical Cation Scavenging Activity.* The ABTS radical cation decolorization test is a spectrophotometric method widely used for assessment of antioxidant activity of various substances. The experiment was carried out using an improved ABTS decolorization assay. In brief, ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation 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 16 h before use. The radical was stable in this form for more than 2 days when stored in the dark at room temperature. For the study of *H. lupulus* extracts, the $ABTS^{\bullet+}$ was diluted with ethanol to an absorbance of 0.700 ± 0.020 at 734 nm and equilibrated at 30 °C. After addition of 2.0 mL of diluted $ABTS^{\bullet+}$ solution (A = 0.700 ± 0.020) to 50, 75, and 100 μL of sample solutions the absorbance reading was taken exactly after 4 min, appropriate solvent blank was run in each assay. All experiments were carried out in triplicate. The percentage inhibition of absorbance at 734 nm is calculated and plotted as a function of concentration of antioxidants.[24] The radical scavenging activity was calculated by formula 1

$$\%\ \text{Inhibition} = [(A_B - A_A)/A_B] \times 100 \quad (1)$$

where A_B = absorption of blank sample and A_A = absorption of tested extract solution.

Ferric-Reducing Antioxidant Power (FRAP) Assay. The FRAP assay was carried out according to the procedure of Benzie and Strain with slight modification. The principle of this method is based on the reduction of a Fe^{3+}-TPTZ to its ferrous, colored form Fe^{2+}-TPTZ in the presence of antioxidants. Briefly, the working FRAP reagent was freshly prepared by mixing together 10 mM TPTZ and 20 mM ferric chloride in 0.25 M acetate buffer, pH 3.6. Plant sample (500 μL) was added to 3500 μL of water followed by 1 mL of FRAP reagent at 1 min intervals. The absorbance of the reaction mixture was then recorded at 593 nm after 4 min against blank. A standard curve was prepared using various concentrations of $FeSO_4.7H_2O$ (r = 0.9753). In this assay, the reducing capacity of the plant extracts tested was calculated with reference to the reaction signal given by a Fe^{2+} solution. FRAP values were expressed as mmol Fe^{2+}/g of sample. All measurements were done in triplicate.[25]

DPPH Radical Scavenging Activity. Radical scavenging activity of extracts against stable $DPPH^{\bullet}$ was also determined spectrophotometrically. When $DPPH^{\bullet}$ reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The change in color (from deep-violet to yellow) was measured at 517 nm. The method used by Takao

et al. was adopted with suitable modifications.[20,21] DPPH (4 mg) was dissolved in MeOH (50 mL) to obtain a concentration of 80 μg/mL.

Quantitative Assay. The extracts (a–h) of *H. lupulus* were dissolved in MeOH to obtain a concentration of 10 mg/mL. Dilutions were made to obtain concentrations of 1, 1×10^{-1}, 1×10^{-2}, 1×10^{-3}, and 1×10^{-4} mg/mL. Diluted solutions (1.00 mL each) were mixed with DPPH (1.00 mL) and allowed to stand for 30 min for any reaction to occur. The UV absorbance was recorded at 517 nm. The experiment was performed in triplicate, and the average absorption was noted for each concentration. The same procedure was followed for the positive standard (quercetin). The percentage of inhibition of DPPH was calculated using formula 1 above. Trolox was also used as a reference in this assay. A standard curve (r = 0.997) was obtained using different concentrations (0–1 mg/mL) of Trolox standard solution. The absorbance of the extract was compared to that of the Trolox standard, and the results of the extracts were expressed as microgram Trolox equivalents per gram of fresh weight of sample (μg TE/g sample).[26]

(CUPRAC) Cupric Reducing Antioxidant Power Assay. The CUPRAC assay was carried out according to the procedure of Apak et al. The CUPRAC method is comprised of mixing the antioxidant solution (directly or after acid hydrolysis) with a copper(II) chloride solution, a neocuproine alcoholic solution, and an ammonium acetate aqueous buffer at pH 7 and subsequently measuring the developed absorbance at 450 nm after 30 min (normal measurement). A 1.0×10^{-2} M copper(II) chloride solution was prepared from $CuCl_2 \cdot 2H_2O$ (0.4262 g) dissolved in H_2O and diluted to 250 mL with additional water. Ammonium acetate (NH_4Ac) buffer at pH 7.0 was prepared by dissolving NH_4Ac (19.27 g) in water and diluting to 250 mL. Neocuproine (Nc) solution (7.5×10^{-3} M) was prepared by dissolving Nc (0.039 g) in 96% EtOH and diluting to 25 mL with ethanol. All hydrophilic polyphenolic compounds and vitamin solutions were freshly prepared in 96% EtOH at 1 mM (1.0×10^{-3} M) concentration prior to measurement. To a test tube were added Cu (II), Nc, and NH_4Ac buffer solutions (1 mL each). Plant sample (or standard) solution (x mL) and H_2O ($1.1 - x$) mL were added to the initial mixture so as to make the final volume 4.1 mL. The tubes were stoppered, and after 1/2 h, the absorbance at 450 nm (A_{450}) was recorded against a reagent blank. The UV–vis spectrophotometer used was Perkin-Elmer lamda 25. The standard calibration curves of each antioxidant compound were constructed in this manner as absorbance versus concentration, and the molar absorptivitiy of the CUPRAC method for each antioxidant was found from the slope of the calibration line concerned. The scheme for normal measurement of hydrophilic antioxidants can be summarized as follows:

1 mL of Cu (II) + 1 mL of Nc + 1 mL of buffer + x mL of antioxidant solution + ($1.1 - x$) mL of H_2O; total volume = 4.1 mL, measure A_{450} against a reagent blank 30 min after reagent addition.[27]

Preparation of Test Solution for LC–MS/MS. A total of 100 mg of extracts were dissolved in 5 mL of ethanol–water (50:50 v/v) in a volumetric flask, from which 1 mL was transferred into another 5 mL of volumetric flask. Then, 100 μL of curcumin was added and diluted to the volume with ethanol–water (50:50 v/v). From the final solution 1.5 mL of aliquot was transferred into a capped auto sampler vial and 10 μL of sample was injected onto LC column. The samples in the autosampler were kept at 15 °C during the experiment.[28]

Instruments and Chromatographic Conditions. Experiments were performed by a Zivak HPLC and Zivak Tandem Gold Triple quadrupole (Istanbul, Turkey) mass spectrometer equipped with a Macherey-Nagel Nucleoder C18 Gravity column (125 × 2 mm i.d., 5 μm particle size). The mobile phase was composed of methanol (A, 0.5% formic acid) in water (B, 0.5% formic acid), the gradient program of which was 0–1.00 min 50% A and 50% B, 1.01–30.00 min 100% A, and finally 30.01–35.00 50% A and 50% B. The flow rate of the mobile phase was 0.3 mL/min, and the column temperature was set to 30 °C. The injection volume was 10 μL and the concentrations of extracts were 1 mg/mL.[28]

LOD and LOQ. LOD and LOQ of the LC–MS/MS methods for the reported compounds were found to be 0.5–50 μg/L. The limits of the quantification (LOQs) were determined to be 10x the S/N for the above concentrations.

LC–MS/MS Analysis. The optimum mobile phase solution was determined to be a gradient of acidified methanol and water system, and good ionization has been obtained by ESI source using a tandem triple quadrupole mass spectrometry system, details of experimental parameters are also given in previous studies.[28,29] The LOD and LOQ of this method have been determined in the range of 0.5–50 to 5.0–500 μg/L, respectively. The whole validation procedure and uncertainty assessment of the method were reported in the literature. The concentration of the compounds in the plant extract, expressed in μg/L within the linear range, was obtained from the linear regression equation of each compound.[28]

GC–MS Analysis. Helium was used as carrier gas at a constant flow rate of 1 mL/min. A total of 1 μL of sample was injected. The GC temperature program was set as follows; 50 °C hold for 5 min, ramp to 250 °C at 5 °C/min and hold for 10 min. The temperature of the MS transfer line was set at 230 °C. Using scan mode a mass range from 50 to 650 m/z. Used column, DB5, 30 m, 0.25 mm ID, 0.25 μm. Thermo Scientific TSQ GC–MS was used in this study.

Statistical Analysis. Results were expressed as mean ± standard error. Correlation between phenolic contents and antioxidant activity was established by regression analysis.

RESULTS AND DISCUSSION

Solvent Extraction. In this study, the effect of various solvents on the Soxhlet extraction of phenolics from cones of *H. lupulus* was investigated. Active extracts were determined by the DPPH solution as shown in Table 1. Dichloromethane (b) and ethylacetate (c) extracts did not show antioxidant activity by the DPPH assay. It was observed that 25% aqueous ethanolic extract (h) was found to be most efficient for extraction of phenolics in comparison to other solvents (Table 1). From these results, it was clear that the addition of some amount of water enhances the extraction efficiency. The reason for the extraction efficiency with aqueous solvents is primarily due to the water-soluble nature of plant phenolics enhanceds by the presence of solvent which facilitates solubilization through penetration in plant cell structure.[30]

Table 1. Total Phenolic Contents in Different Solvent Extracts

extract	total phenolic contents (TPC)[a]
hexane (a)	1344 ± 31.52
methanol-3 (d)	4244 ± 88.74
methanol-1 (e)	8343 ± 158.39
acetone (f)	1338 ± 34.29
methanol-2 (g)	688 ± 18.39
25% aqueous ethanol (h)	9079 ± 187.83

[a]Data expressed as mg of ferulic acid equivalent (FAE)/100 g of extract.

Total Phenolic Content. The total phenolic content of the plant extracts is shown in Table 1. Among all the *H. lupulus* extracts, 25% aqueous ethanol extract (h) had the highest phenolic content (9079 ± 187.83 mg/100 g extract), followed by methanol-1 (e) (8343 ± 158.39 mg/100 g), methanol-3 (d) (4244 ± 88.74 mg/100 g), *n*-hexane (a) (1344 ± 31.52 mg/100 g), acetone (f) (1338 ± 34.29 mg/100 g), and methanol-2 (g) (688 ± 18.39 mg/100 g). A similar highest result of total phenolic content was also reported previously from selected vegetables such as spinach, cabbage, swamp cabbage, kale and shallots.[23] Finding antioxidant activity in the *n*-hexane extract which includes major constituents of many food and dietary

supplements is going to be interesting among the other plants. Total phenolic content of 80% aqueous methanol extract of hop cones prepared in test tubes with 1.0 g plant material and 10 mL solvent under sonication, was reported as 7.14 ± 0.16 GAE/100 g of dry weight. The results of ABTS, DPPH, and FRAP assays were reported, respectively, such as 10.8 ± 0.11, 83.2 ± 2.00, and 50.3 ± 2.34 μM trolox/100 g dw.[44] It is clear that the phenolic contents in all Soxhlet extracts of naturally groving hops from Table 1 were higher than the above-reported results. Several studies have reported on the constituents of *H. lupulus*.[3,31]

Antioxidant Activity of *H. lupulus*. Radical scavenging capacities were determined using the DPPH, ABTS, CUPRAC and FRAP assays. In the DPPH assay, inhibitory concentration (IC_{50}) of plant extracts varied from 8.67 to 91.63 μg/mL. The *n*-hexane extract showed the highest antioxidant capacity (8.67 μg/mL), followed by hop methanol-1 extract (9.32 μg/mL), methanol-3 extract (44.36 μg/mL), methanol-2 extract (49.25 μg/mL) and 25% aqueous ethanol extract (78.7 μg/mL). Acetone extract showed the lowest antioxidant capacity (91.63 μg/mL). Results are shown in Table 2. DPPH values ranged from 14.95 to 157.98 μg Trolox equivalent per gram of sample as shown in Table 3.

Table 2. Radical Scavering Capacities and Quercetin Equivalent Flavonoid Concentration of *H. lupulus* Extracts

extract	DPPH IC_{50} (μg/mL)	ABTS inhibition %	CUPRAC (QREFC)[a]
hexane (a)	08.67 ± 0.07	30.34 ± 0.04	1.36 ± 0.01
methanol-3 (d)	44.36 ± 1.62	60.51 ± 0.12	1.88 ± 0.18
methanol-1 (e)	09.32 ± 0.13	51.70 ± 0.09	2.10 ± 0.21
acetone (f)	91.63 ± 1.87	37.00 ± 0.20	1.46 ± 0.51
methanol-2 (g)	49.25 ± 0.17	50.30 ± 0.94	1.26 ± 0.46
25% aqueous ethanol (h)	78.70 ± 0.34	48.30 ± 0.73	1.11 ± 0.08

[a]Quercetin equivalent of flavonoid concentration.

In the ABTS assay, values ranged from 30.34 to 60.51% for 100 μL of 1 mg/mL sample solution. Methanol-3 extract possessed the highest antioxidant capacity (60.51% of ABTS inhibition) followed by methanol-1 extract (51.7%), methanol-2 extract (50.3%), 25% aqueous ethanol extract (48.3%), acetone extract (37%). Inhibition values of ABTS assay are shown in Table 2. According to the DPPH assay, *n*-hexane extract showed the lowest antioxidant capacity (30.34%). TEAC values varied from 4.48 to 7.35 mM Trolox equivalent per gram of sample, which showed the highest antioxidant capacity was methanol-2 extract (7.35 mM/g), followed by methanol-1 extract (7.22 mM/g), 25% aqueous ethanol extract (6.91 mM/g), methanol-3 extract (6.34 mM/g), acetone extract (5.25 mM/g), and *n*-hexane extract (4.48 mM/g). The contents in the extracts positively correlate with their antiradical activity approving join to the radical scavenging activity of the extracts (Figure 1a).

In the CUPRAC assay, values of extracts which ranged from 1.11 to 2.1 as quercetin equivalent of flavonoid concentration (QREFC), were measured as methanol-1 > methanol-3 > acetone > hexane > methanol-2 > 25% aqueous ethanol and $TEAC_{CUPRAC}$ values varied from 1.64 to 3.15 mmol Trolox equivalent per gram of sample. According to reported study of Apak et al., CUPRAC values were highest for Ceylon blended ordinary tea (4.41 mmol TE/g), green tea with lemon (1.61 mmol TE/g), English breakfast ordinary tea (1.26 mmol TE/g) and green tea (0.94 mmol TE/g). CUPRAC results of *H. lupulus* extracts were higher than the reported results of Apak et al. for green tea with lemon, English breakfast ordinary tea, and green tea extracts.[27]

Ferric reducing antioxidant capacities of extracts tested varied from 0.3 to 1.56 mM $FeSO_4$ /g. The highest activity was showed by methanol-3 (1.56 ± 0.38 mM Fe^{2+}/g sample) and methanol-2 (1.5 ± 0.09 mM Fe^{2+}/g sample) extracts. Hexane extract showed the lowest antioxidant activity with this method (0.3 ± 0.02 mM Fe^{2+}/g sample). Due to the scavenging of DPPH cation radicals in the DPPH assay and reduction of ferric ion in the FRAP assay, the results of these assays were significiantly differences in all extracts tested. FRAP assay is the only assay that directly measures antioxidants or reductants in a sample. In FRAP assay, the results were explained as the combined concentrations of all electron-donating reductants which take place in the samples in a class of sample plants. For instance, while *n*-hexane extract of *H. lupulus* exhibit the strong antioxidant activity in the DPPH assay, there is a antipodal action in the FRAP assay, so the correlation was found considerably reverse between these two assays. The results are shown in Tables 2 and 3 and Figure 1b.

The chemical complexity of extracts could lead to scattered results, depending on the test employed. Therefore, an approach with multiple assays screening is highly advisable.[32−34] Thus, the extracts were subjected to four different antioxidant assays which used CUPRAC, $ABTS^{\bullet+}$, $DPPH^{\bullet}$, and FRAP methods. According to these assays, all of the extracts of cones of *H. lupulus* obtained using Soxhlet were shown to exhibit significant inhibitory activity against free radicals.

Phenolic substances can be extracted from plant material using a sequence of solvents with different polarity. Among antioxidant phenolics, certain classes of compounds such as phenolic acids, hydroxycinnamic acids, flavonoids, and carotenoids require a decreasing order of solvent polarity for

Table 3. Trolox Equivalents of Antioxidant Activities of the Extracts of *H. lupulus* as Measured by ABTS, DPPH, CUPRAC, and FRAP Assays

extract	DPPH[a]	CUPRAC[b]	ABTS[c]	FRAP[d]
hexane (a)	14.95 ± 0.03	2.52 ± 0.01	4.48 ± 0.13	0.30 ± 0.02
methanol-3 (d)	76.48 ± 0.12	1.83 ± 0.10	6.34 ± 0.26	1.56 ± 0.38
methanol-1 (e)	16.07 ± 0.04	1.64 ± 0.54	7.22 ± 0.14	1.38 ± 0.78
acetone (f)	157.98 ± 1.07	2.36 ± 0.31	5.25 ± 0.18	0.81 ± 0.16
methanol-2 (g)	84.91 ± 1.44	2.73 ± 0.27	7.35 ± 0.03	1.50 ± 0.09
25% aqueous ethanol (h)	135.69 ± 1.23	3.15 ± 0.44	6.91 ± 0.41	1.34 ± 0.11

[a]Data expressed as μg of Trolox equivalent/g of plant material. [b]Data expressed as mmol of Trolox equivalent/g of plant material. [c]Data expressed as mM of Trolox equivalent. [d]Data expressed as mM of Fe^{2+} equivalent/g of plant material.

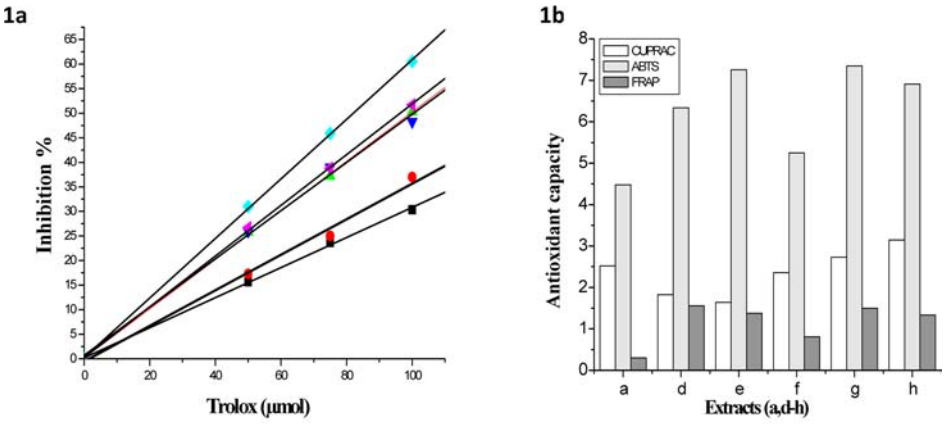

Figure 1. Correlation between inhibitions measured by ABTS assay in different extracts of *H. lupulus* ■, hexane; ● acetone; ▲, methanol-2 (g); ▼, 25% aqueous ethanol; ♦, methanol-3 (d); left pointing solid triangle, methanol-1 (e) (1a). Antioxidant capacity in different extracts of *H. lupulus* measured by CUPRAC, ABTS, and FRAP assays (1b).

Table 4. LC−MS/MS Parameters of Selected Compounds and Amount of Antioxidants in *H. lupulus* Extracts in μg/g Concentration

						amounts of antioxidants in the plant extracts (μg/g)[b]					
compounds	parent ion (*m*/*z*)	daughter ion (*m*/*z*)	collision energy (V)	LOD/LOQ (ppb)	U_{95} (%)	a	d	e	f	g	h
curcumin[a]	367	216.4	10	—	—	—	—	—	—	—	—
kaempherol-3-*O*-glucoside	447	284	20	20/60	3.2	163	213	185	—	—	128
ascorbic acid	175	114.6	12	15/50	2.28	20	13	19	186	28	—
gallic acid	169	124.6	10	0.4/1.4	1.87	185	—	—	174	—	157
epigallol	305	124	25	15/45	2.1	26	—	—	—	—	—
quercetin	301	178.6	10	1.2/4.2	1.64	—	81	72	—	—	46
ellagic acid	301	150.5	10	0.2/1	2.53	—	56	59	—	—	48
p-coumaric acid	163	118.7	10	0.2/1	3.98	—	—	—	—	125	—
pyragallol	125	78.7	20	1.4/5	2.06	—	—	—	—	—	52
ferulic acid	193	177.5	10	0.2/0.8	3.97	—	—	—	—	84	—

[a]It was used as internal standard (35,36). [b]The uncertainty of results should be calculated according to ref 28.

extraction, respectively, although suitable solvent combinations may be tailored for specific purposes.[35,36] Although it is difficult to define a universally acceptable solvent, 80% MeOH and 70% EtOH are generally the most preferred solvents for phenolics extraction from plants. Due to the diversity of phenolic antioxidant phytochemicals in botanicals, certain compromises have to be made in solvent selection.[27]

In accordance with these reports, we tried different solvent systems with changing polarity as mentioned above. There are the noticeable discrepancies between the antioxidant assay values of the extracts, so there is no correlation between the antioxidant results of extracts which have illustrated the different properties in the antioxidant assays. Therefore, it is possible to say that by changing solvent, active components of the extracts will have differences which is shown in Table 3.

LC−MS/MS Analysis of Extracts. The recent focus of interest on phenolic acids stems from their potential protective role, through ingestion of fruits and vegetables, against oxidative damage diseases such as coronary heart disease, stroke, cancers, cardiovascular diseases, and inflammation. Also, researchers and food manufacturers are interested in phenolic acids because of their strong antioxidant properties, abundance in the human diet, and probable role in the prevention of various diseases associated with oxidative stress.[28] Phenolic acids are regarded as one of the functional food components in fruits and are thought to contribute to the health effects of plant-derived products by scavenging free radical species, inhibiting free radical formation, and preventing oxidative damage to DNA.[28,37]

Our study provides valuable information on the antioxidant capacity of *H. lupulus*. Polyphenols, the large group of phytochemicals, are known to act as antioxidants. The content of phenolic compounds (mg/100 g) of the extracts was expressed as milligram of ferulic acid equivalents (FAE; Table 1). Phenolic compounds are likely to contribute to the radical scavenging activity of these plant extracts. Phenolic acids are plant metabolites widely spread throughout the plant kingdom. The recent focus of interest on phenolic acids stems from their potential protective role, through ingestion of fruits and vegetables, against oxidative damage diseases such as coronary heart disease, stroke and cancers. The profile of phenolic acids in *H. lupulus* extracts was analyzed by LC−MS/MS. Referring to Table 4 and Figures 2 and 3; it is clearly shown that kaempherol 3-*O* glucoside, quercetin, ascorbic acid, ferulic acid, gallic acid, ellagic acid, *p*-coumaric acid, epigallol, and pyrogallol are the predominant phenolic compounds identified in some of the extracts. Structures of these components are given in the Supporting Information, Figure S4.

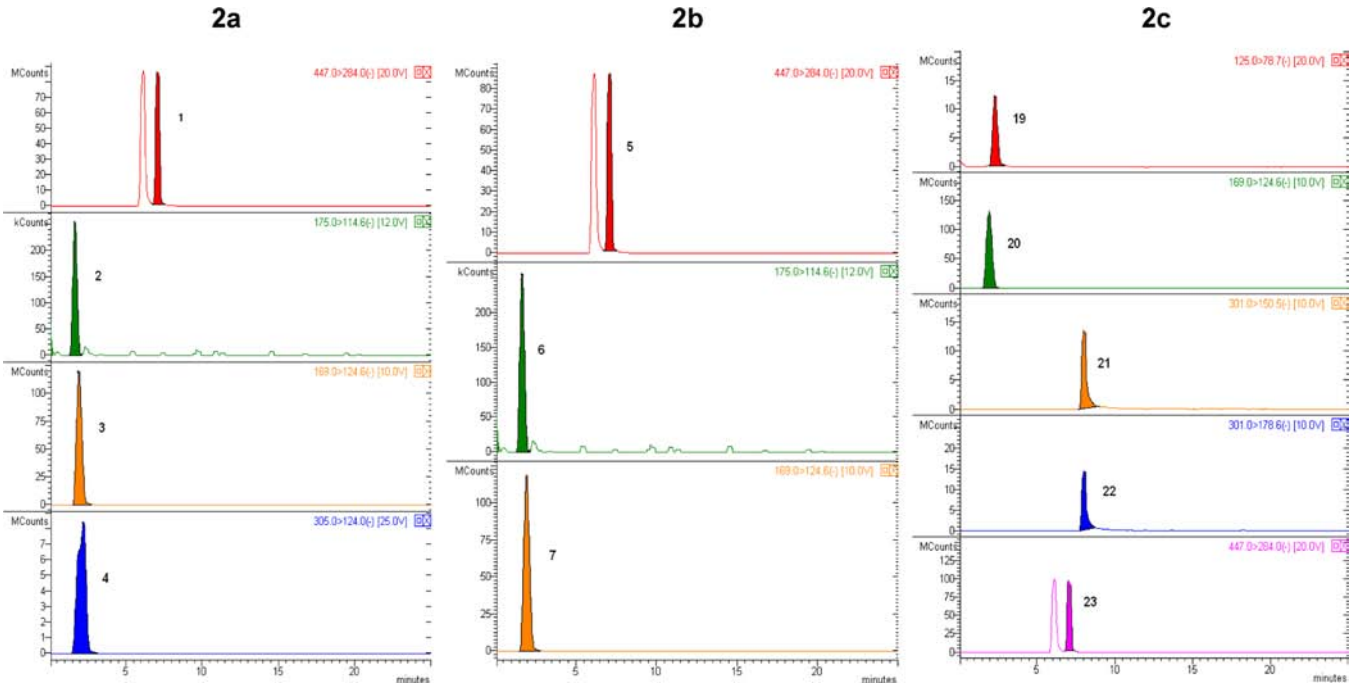

Figure 2. Chromatograms of antioxidants by LC−MS/MS of *H. lupulus* (2a) *n*-hexane extract, (2b) acetone extract, and (2c) 25% aqueous ethanol extract. Kaempherol-3-*O*- glucoside (1, 5, 23), ascorbic acid (2, 6), epigallol (4), gallic acid (3, 7, 20), pyragallol (19), ellagic acid (21), and quercetin (22).

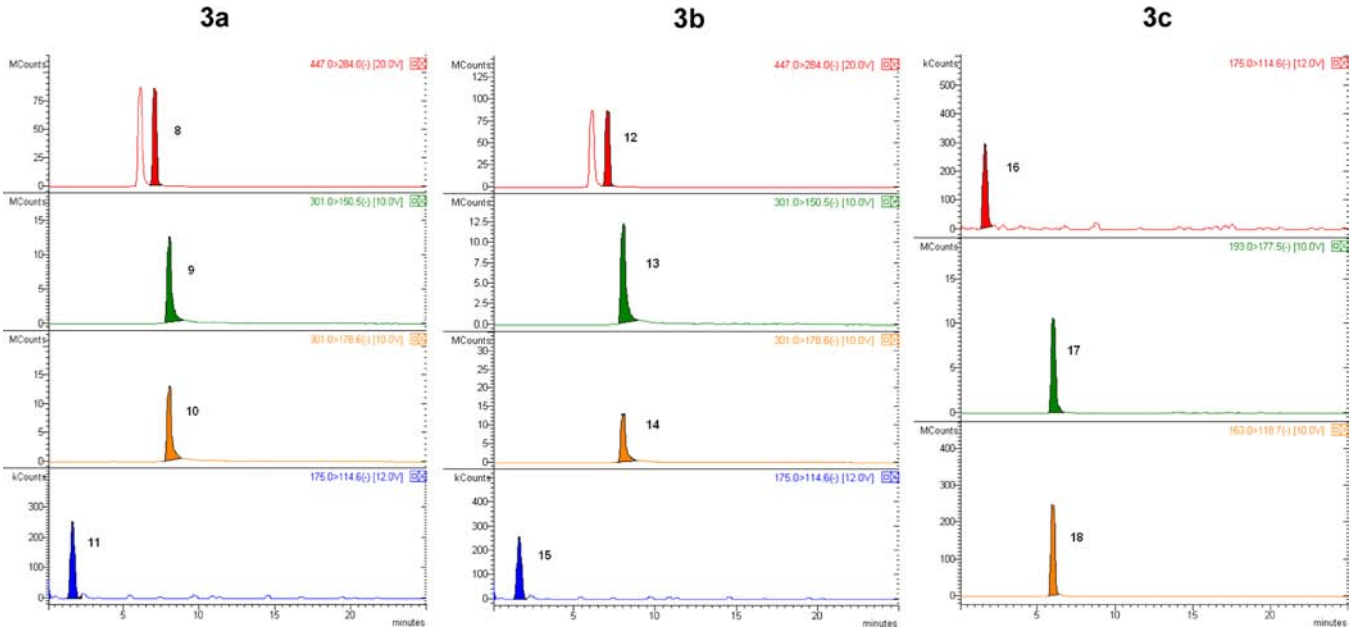

Figure 3. Chromatograms of antioxidants by LC−MS/MS of *H. lupulus* (3a) methanol-1 extract, (3b) methanol-3 extract, and (3c) methanol-2 extract. Kaempherol-3-*O*-glucoside (8, 12), ellagic acid (9, 13), quercetin (10), ascorbic acid (11, 15, 16), quercetin (14), ferulic acid (17), and *p*-coumaric acid (18).

Chromatograms of identified compounds from methanol-1 extract, ferulic acid, humulone, lupulone, cohumulone, colupulone, adhumulone, adlupulone, xanthohumol, 6-prenylnaringenin, 8-prenylnaringenin, isoxanthohumol, microbial transformation product, 2″,3″-dihydroxanthohumol, 2″,2″-dimetil-3″,4″-dihidropiran-[2″,3″:3′,4′]-2′,4-dihydroxy-6′-methoxycalcone or chlorogenic acid, myrcene, caryophyllene or farnesene, valerinic acid and quercitrin by LC−MS (SIM mode) of *H. lupulus* are given in the Supporting Information, Figure S2, and detection of these compounds was optimal under negative ion SIM mode. In this analysis coinjection was just applied to kaempferol, epigallol, quercetin, naringenin and naringin. The clear structures of these compounds are given in the Supporting Information, Figure S3.

GC−MS Analysis of Extracts. The characterization of the acide esters in the extracts were carried out by GC−MS, in addition, ubiquitous compounds of *H. lupulus* plant such as cohulupone, eupulone and *β*-lupulic acid (lupulone) were

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Table 5. Identified Compounds from *H. lupulus* Extracts by GC−MS[a]

					extract (peak area %)					
no.	RT	compounds	MF	MW	g	e	d	h	a	f
1	10.14	glutaric acid, dimethyl ester	$C_7H_{12}O_4$	160.17	—	0.8	—	0.4	—	—
2	11.48	benzaldehyde dimethyl acetal	$C_9H_{12}O_2$	152.19	—	0.7	—	0.4	0.4	0.5
3	16.13	5-hexenoic acid, 5-bromo-, methyl ester		—	—	0.6	—	—	—	—
4	22.78	nonanedioic acid, dimethyl ester	$C_{11}H_{20}O_4$	216.27	2.2	0.8	0.9	0.5	—	—
5	25.51	selinene	$C_{15}H_{24}$	204.35	—	—	—	—	0.6	—
6	26.86	myristic acid, methyl ester	$C_{15}H_{30}O_2$	242.39	1.3	1.2	1.2	1.3	0.8	0.7
7	27.61	indole-3-butyric acid, methyl ester	$C_{13}H_{15}NO_2$	217.26	—	—	6.4	—	—	—
8	28.93	pentadecanoic acid, methyl ester	$C_{16}H_{32}O_2$	256.42	—	—	—	—	0.6	0.5
9	29.12	benzoic acid, phenyl ester	$C_{13}H_{10}O_2$	198.22	21.7	—	—	—	—	—
10	29.33	ferulic acid, methyl ester	$C_{11}H_{12}O_4$	208.21	6.6	—	2.3	—	—	—
11	30.44	7,10,13-hexadecatrienoic acid, methyl ester	$C_{17}H_{28}O_2$	264.40	5.5	—	—	—	—	—
12	30.54	11-hexadecenoic acid, methyl ester	$C_{17}H_{32}O_2$	268.43	—	0.4	—	—	—	—
13	31.05	palmitic acid, methyl ester	$C_{17}H_{34}O_2$	270.45	20.1	11.5	29.1	9.1	9.7	11.8
14	31.34	cohulupone	$C_{19}H_{26}O_4$	318.41	—	3.0	—	—	—	—
15	32.22	2-benzoyloxysuccinic acid, dimethyl ester	$C_{13}H_{14}O_6$	266.24	2.2	—	—	—	—	—
16	32.67	3,4,5-trimethoxy, benzenebutanoic acid	$C_{13}H_{16}O_6$	268.26	—	—	2.9	—	—	—
17	32.87	heptadecanoic acid, methyl ester	$C_{18}H_{36}O_2$	284.47	—	3.9	1.7	—	2.0	3.4
18	33.84	phytyl methyl ether		—	4.4	4.9	—	5.8	8.1	9.9
19	33.98	arachidonic acid, methyl ester	$C_{21}H_{34}O_2$	318.49	—	—	—	2.6	—	—
20	34.05	2-phenazinecarboxylic acid, methyl ester	$C_{14}H_{10}N_2O_2$	238.24	2.6	—	—	—	—	—
21	34.27	linoleic acid, methyl ester	$C_{19}H_{34}O_2$	294.47	8.8	20.0	23.2	16.8	16.2	19.4
22	34.30	linolenic acid, methyl ester	$C_{19}H_{32}O_2$	292.46	17.5	—	18.9	15.1	20.3	19.5
23	34.38	oleic acid, methyl ester	$C_{19}H_{36}O_2$	296.49	—	19.5	1.2	16.1	18.6	18.2
24	34.74	stearic acid, methyl ester	$C_{19}H_{38}O_2$	298.5	2.6	4.9	6.4	3.6	6.1	9.2
25	38.13	eicosanoic acid, methyl ester	$C_{21}H_{42}O_2$	326.56	1.8	4.0	2.9	5.8	6.5	4.6
26	39.42	2,4,6-tritert-butyl benzoic acid	$C_{19}H_{30}O_2$	290.44	1.1	7.9	—	—	4.0	—
27	40.63	*β*-lupulic acid	$C_{26}H_{38}O_4$	362.58	0.9	7.9	—	18.3	—	—
28	41.29	tricosanoic acid	$C_{23}H_{46}O_2$	354.61	0.7	4.0	2.9	3.3	6.1	2.3
29	41.39	eupulone		—	—	2.0	—	—	—	—
30	44.16	lignoceric acid, methyl ester	$C_{25}H_{50}O_2$	382.66	—	2.0	—	0.9	—	—

[a]RT: retention time, MF: molecular formula, MW: molecular weight.

identified with this analysis. Cohulupone was determined from methanol-1 extract with 20% relative abundance at 31.34 min. *β*-Lupulic acid was determined from methanol-2, methanol-1 and 25% aqueous ethanol extracts, with about 20%, 50% and %100 relative abundance, and selinene (3%) and eupulone (30%) were shown in *n*-hexane and methanol-1 extracts, respectively. The various phytochemicals which contribute to the medicinal activities of the plant were shown in Table 5.

The GC−MS chromatograms of the extracts of *H. lupulus* were presented (Supplementary Figure S1). The presence of various bioactive compounds in the *H. lupulus* justifies the use of plant for various diseases by traditional practitioners. From the results, it could be concluded that *H. lupulus* contains various bioactive compounds. Therefore, it is recommended as a plant of phytopharmaceutical importance.

Antioxidants such as phenolics and flavonoids, present in fruits and vegetables, have been positively correlated to the reduced incidence of heart disease, some cancers, and age-related degenerative diseases. *H. lupulus* plant investigated in this study were shown to be a novel rich source of polyphenolic and antioxidant compounds. This study demonstrated that plant cones has high potential value for food suplementary as well as the beer industry because of their high polyphenolic contents. The polyphenolics are a structurally diverse class of plant secondary metabolites. In general, they possess an aromatic ring bearing one or more hydroxyl substituent.[28,38]

Today, a wide range of over-the-counter preparations containing hop extracts or hop-derived products is available on the market, in particular for use in the phytotherapy of sleep disorders or pain relief and in postmenopausal therapy.[39−42] Hop extracts are the major constituents of many food and dietary supplements with claim of "breast enhancement", but also in this case properly controlled clinical trials supporting the use of hops for their estrogenic properties are still lacking.[43] The use of chemically characterized hop extracts for biological assays and for clinical trials is the right approach to study their pharmacokinetic and pharmacological profile and to perform comparative studies, with the aim to validate the above-mentioned properties of hops. There are still a lot of works to be done in order to achieve a reliable standardized product and to link it to a specific biological activity and to specific therapeutic applications.[3] It is known that maximum secondary metabolite concentrations depends on region and climate differences. Therefore, this paper reports apparently the first comparative study emphasize the antioxidant activities of the extracts of naturally growing plant in Turkey. As far as known that it is the first time, CUPRAC assay was applied to *H. lupulus* extracts. Due to QREFC (1.11 to 2.1) and $TEAC_{CUPRAC}$ (1.64 to 3.15) values of methanol-1 > methanol-3 > acetone > hexane > methanol-2 > 25% aqueous ethanol extracts, qualitative analysis of methanol-1 extract of hops was run by LC-MS (SIM mode) and coinjection. Quantitative analysis of some of the

major components of extracts by LC−MS/MS are the first time run in this paper.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figure S1. Chromatograms of extracts of *H. lupulus* acquired with GC−MS analysis: (a) methanol-1, (b) methanol-2, (c) methanol-3, (d) ethanolic, (e) *n*-hexane, and (f) acetone. Figure S2. Chromatograms of identified compounds by LC−MS (SIM mode) of *H. lupulus* methanol-1 extract (1), ferulic acid (2), humulone (3), lupulone (4), cohumulone (5), colupulone (6), adhumulone (7), adlupulone (8), xanthohumol (9), naringenin (10), 6-prenylnaringenin (11), 8-prenylnaringenin (12), isoxanthohumol (13), microbial transformation product (14), 2″,3″-dihydroxanthohumol (15), 2″,2″-dimetil-3″,4″-dihidropiran [2″,3″:3′,4′]2′,4-dihidroksi-6′-methoxychalcone or chlorogenic acid (16), kaempherol (17), unknown (18), quercetin (19), myrcene (20), caryophyllene or farnesene (21), valerinic acid (22), astragalin or quercitrin (23), and naringin (24). Figure S3. Structures of identified compounds by LC−MS (SIM mode) of *H. lupulus* methanol-1 extract. Names of the compounds were given in Figure S2. Figure S4. Structures of quantitatively identified compounds by LC−MS/MS of *H. lupulus*. Kaempferol-3-*O*-glucoside (25), ellagic acid (26), pyrogallol (27), ascorbic acid (28), *p*-coumaric acid (29), and gallic acid (30). Ferulic acid (2) and quercetin (19) were quantitatively and qualitatively identified by LC−MS/MS and LC−MS (SIM mode), respectively (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

ROS, reactive oxygen species; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2′-azinobis (3-ethylbenzothiazoline 6-sulfonate); FRAP, ferric reducing antioxidant potential; CUPRAC, cupric reducing antioxidant capacity; TPTZ, 2,4,6-tripyridyl-s-triazine; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Neocuproin, 2,9-dimethyl-1,10-phenanthroline; XN, ksanthohumol; IXN, iso-ksanthohumol; 6-PN, 6-prenylnaringenin; 8-PN, 8-prenylnaringenin; Fe^{3+}-TPTZ, ferric 2,4,6-tris (2-pyridyl)-1,3,5-triazine

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