CHEMICAL CONSTITUENTS, QUANTITATIVE ANALYSIS AND ANTIOXIDANT ACTIVITIES OF ECHINACEA PURPUREA (L.) MOENCH AND ECHINACEA PALLIDA (NUTT.) NUTT.

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ABSTRACT

Echinacea is valuable for its pharmaceutical, medicinal and agricultural properties. Flowers and leaves of Echinacea purpurea (L.) Moench and Echinacea pallida (Nutt.) Nutt. were extracted with methanol, filtered, and solvents were removed by rotary evaporator to get four separate extracts. The flowers and leaves of both plants were boiled in water then extracted with ethyl acetate to achieve another four extracts. Quantifications of chemical constituents of extracts were determined by TOF-LC/MS. The main compound of methanol extracts of E. purpurea and E. pallida leaves and flowers was cichoric acid. Caffeic acid was the chief compound of water extracts of both plant leaves and flowers. The antioxidant activities including DPPH free radical scavenging, ABTS cation radical scavenging and reducing power were assayed and structure–activity relationships were postulated. Water extracts of both Echinacea species of flowers and leaves revealed excellent antioxidant activities.

PRACTICAL APPLICATIONS

Echinacea, which is a medicinal and aromatic plant, has been used for traditional medicine in many countries. The water extract of Echinacea purpurea (L.) Moench and Echinacea pallida (Nutt.) Nutt. exhibited excellent antioxidant activities; therefore, these Echinacea species can be used as natural agents in food and pharmaceutical industries.

INTRODUCTION

The genus Echinacea pertains to an herb and is a perennial purple coneflower distributed through North America and Europe belonging to the Asteraceae family. There are 9 species, 11 taxa and 2 botanical varieties of which chiefly Echinacea purpurea (L.) Moench and Echinacea pallida (Nutt.) Nutt. have been used as traditional medicine. (MacGregor 1968; Chen et al. 2005). Echinacea has long been used as an herbal medicine for many remedies in many countries (Nilanthi et al. 2009). It has constantly been one of the best selling genera in the American herb market (Qu et al. 2005). Because of the high demand for Echinacea, harvest has brought about a swift development of Echinacea cultivation throughout the world (Li 1998). Its species include caffeic acid derivatives, polysaccharides and lipophilic alkamides responsible for medicinal properties (Wills and Stuart 1999).

Among the Echinacea species, E. purpurea is a significant medicinal plant that achieved attention all over the world due to its anti-inflammatory, antibacterial and lymphocyte activities as well as its enhancement of the immune system (Weber et al. 2005). It is also used for the treatment of various illnesses such as typhoid fever and respiratory infections, and it activates the human phagocytic function and prevents the development of benign prostate hyperplasia.
(See et al. 1997). E. pallida is as important as E. purpurea and other species for pharmaceutical effect consisting of ketoalkenes and ketoalkenynes exhibited the cytotoxic (Chicca et al. 2008), cannabinoid receptor (Egger et al. 2008), acetylcholinesterase, antioxidant (Orhan et al. 2009), and anti-inflammatory (Zhai et al. 2009a). Hexane extract of E. pallida, containing alkylamides and polyacetylenes, displayed significant cytotoxic and pro-apoptotic activities against human colon cancer and pancreas cancer (Chicca et al. 2007). A recent research showed that oral administration of E. pallida extract has the ability to treat wounds at high level of corticosterone in mice (Zhai et al. 2009b).

Recently, Echinacea species, E. purpurea and E. pallida, is one of the largest sectors of several billion dollar herbal medicine market in North America as well as Europe. Echinacea products cost nearly $US330 million in the U.S.A. annually (Foster 1994; Bauer 1999). Echinacea is available in pharmacies and health food stores in North America and Europe as direct pressed juices, freeze-dried ethanolic or hydrophilic extracts, and whole or powdered dried leaves and flowers (Barrett 2003).

Free radicals, reactive oxygen species (ROS), a part of many chemical reactions in eukaryotic cells including superoxide, hydroxyl radical and singlet oxygen, can harm cells then cause disease. Free radicals lead to cardiovascular diseases, DNA damage and cancer (Frohlich et al. 2008). The human body has many defense functions against oxidation, such as antioxidant enzymes and nonenzymatic components. In some cases, natural antioxidant in the body becomes insufficient, and free radicals can damage cell membrane in a chain reaction causing diseases (Chandran et al. 2012). Antioxidants can defend the human body from ROS effects; therefore, many drugs having the antioxidant capacity are used for the treatment of diseases like stroke, diabetes, Alzheimer’s disease and cancer (Je et al. 2005; Wong et al. 2006). Antioxidants, which are important in physiological systems, have been used in the food industry to extend the life of foods (Gulcin 2012). These compounds are oxidized by free radicals which are the main source of deterioration, nutritional losses and discoloration in foods (Jaiswal et al. 2012). Lately, many scientific works have been focused on finding natural antioxidants for use in food of commerce, perfumery and pharmaceutics to replace synthetic antioxidants, which are restricted on account of their carcinogenicity (Madhavi and Salunkhe 1995; Botterweck et al. 2000).

In previous work, we presented the anticancer activities of isolated compounds and methanol extract of E. pallida (Yaglioglu et al. 2013). In this study, we determined the chemical constituents of E. purpurea and E. pallida and investigated antioxidant activities as well as displaying the relationship between the chemical constituents and antioxidant activities.

**MATERIALS AND METHODS**

**Reagents**

Butylated hydroxyanisole, butylated hydroxytoluene, nico-
tinamide adenine dinucleotide, linoleic acid, α-tocopherol, nitroblue tetrazolium, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), 3-(2-pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine) and polyoxyethylene sorbitan monolaurate (Tween-20), and trichloroacetic acid (TCA) were obtained from Sigma (Sigma-Aldrich Chemie GmbH, Sternheim, Germany). Ammonium thiocyanate and solvents were supplied from Merck (Kenilworth, NJ).

**Plant Materials**

The plants were collected from the cultivated “Aromatic and Medicinal Plant” field of Gaziosmanpasa University, Faculty of Agriculture, during the harvest period. The plant materials were identified by Assoc. Prof. Dr. Ahmet Ilicim, Mustafa Kemal University, Faculty of Art and Science, Department of Biology, Hatay, Turkey, where the voucher specimens were deposited (E. purpurea: MKUH 1612, E. pallida: MKUH 1613).

**Extraction**

Two extraction methods were applied for the plant materi-
als. One is direct methanol extraction. In another method, plant materials were boiled in water then aqueous part was extracted with ethyl acetate. In the first method, E. purpurea flowers (EPU flowers), E. purpurea leaves (EPL leaves), E. pallida flowers (EPA flowers) and E. pallida leaves (EPL leaves) (each 200 g) were individually extracted with methanol (3 × 2 L) for a week. After the removal of the solvent by rotary evaporator, four extracts were obtained. In the second method, the plant materials (each 200 g), leaves and flowers of E. purpurea and E. pallida, were boiled in water (2 L) for 2 h, filtered, extracted with ethyl acetate, and then evaporated to achieve four extracts separately. In total, eight extracts were acquired to be analyzed for quantifications of chemical constituents and investigation of antioxidant activities.

**Quantitative Analysis of Chemical Constituents**

TOF-LC/MS was used for quantitative analysis. It was Agilent 6210 with column ZORBAX SB-C18, 4.6 × 100 mm, 3.5 μm, 2.7 mm (Agilent Technologies) with an injection volume of 10 mL. The mobile phase consisted of the eluent A, water with 0.1% formic acid and B, acetonitrile. The flow rate was 0.7 mL/min at 35°C. The gradient...
was calculated using the equation:

\[ \text{Absorbance} = 0.0053 \times \text{Total Phenols} - 0.0059 \]

**Determination of Total Phenolic Content**

Total phenolic contents in *E. purpurea* leaves extract, *E. purpurea* flowers extract, *E. pallida* leaves extract and *E. pallida* flowers extract were determined as micrograms of gallic acid equivalent per gram of extract by equation obtained from standard gallic acid graph:

\[ \text{Absorbance} = 0.0053 \times \text{Total Phenols} - 0.0059 \]

**DPPH Free Radical Scavenging Activity**

DPPH free radical scavenging activity of *E. purpurea* leaves extract, *E. purpurea* flowers extract, *E. pallida* leaves extract and *E. pallida* flowers extract was determined by Blois method (Blois 1958). Briefly, to the extract solution of different concentrations (3 mL, 5–20 mg/mL), DPPH solution was added (0.1 mM, 1 mL). The mixture was shaken and kept at RT for 30 min. The absorbance was measured at 517 nm on a spectrophotometer and the lower absorbance of the reaction mixture revealed the higher free radical scavenging activity. The ability to scavenge the DPPH radical was calculated using the equation:

\[ \text{DPPH}^* \text{ scavenging effect (\%)} = \left[ \left( \text{Ac} - \text{As} \right) / \text{Ac} \right] \times 100 \]

Ac is the absorbance of the control, and As is the absorbance in the presence of samples or standards.

**ABTS**•⁺ Scavenging Activity

ABTS⁺ scavenging activity assay was carried out according to the literature (Re *et al.* 1999). The treatment of 7 mM stock solution of ABTS⁺ (in 20 mM acetate, pH 4.5) with potassium persulfate (2.45 mM) for 15 h at RT in the dark yielded the ABTS radical cation. ABTS⁺ solution was diluted with sodium acetate buffer (20 mM, pH 4.5) (1:75, v/v) to accomplish an absorbance at 734 nm of 0.700 ± 0.02. Then ABTS⁺ solution (1 mL) was added with 3 mL of each extract solution in ethanol at different concentrations (20–80 μg/mL). After 30 min, percentage inhibition was calculated for each concentration at 734 nm relative to a blank absorbance. A standard curve was plotted based on measuring the reduction in absorbance of ABTS⁺ solution against different concentrations of standard. The ABTS⁺ concentration was calculated by a calibration curve, determined by linear regression (r²: 0.9843):

\[ \text{Absorbance} = 4.71 \times [\text{ABTS}^+] + 0.199 \]

The scavenging capability was calculated as the following equation:

\[ \text{ABTS}^+ \text{ scavenging effect (\%)} = \left( 1 - \text{As/Ac} \right) \times 100 \]

where Ac is the initial concentration of ABTS⁺ and As is the absorbance of the remaining concentration of ABTS⁺ in the reaction mixture.

**Reducing Power**

The reducing power of *E. purpurea* leaves extract, *E. purpurea* flowers extract, *E. pallida* leaves extract and *E. pallida* flowers extract was measured by the given method (Oyaizu 1986). To the *Echinacea* extract solutions (5, 10, 20, 40 μg/mL) in 1.0 mL distilled water, phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [K₃Fe(CN)₆] (2.5 mL, 1%) were added. After the incubation of the reaction mixture at 50°C for 30 min, TCA (10%, 2.5 mL) was added, then the mixture was centrifuged for 10 min. The solution of upper layer (2.5 mL) was mixed with distilled water (2.5 mL) and FeCl₃ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm by a spectrophotometer. Higher absorbance of the reaction mixture indicated better reducing power.

**RESULTS AND DISCUSSION**

Quantitative analysis on *Echinacea* species of leaf and flower (*E. purpurea* and *E. pallida*) with different extraction procedures was shown in Table 1. The LC-TOF/MS spectrum which showed the chemical constituents was also displayed along with the standards (Figs. 1–3). Major variations were determined among species, plant parts and extraction procedures. Water extract values of phenolic acids are generally
low except for caffeic acid. However, high values of major components containing cichoric acids were determined in methanol extracts.

Cichoric acid, chlorogenic acid, ferulic acid and rutin in *Echinacea* species had high values in methanol extracts of leaf and flower of both species. In plant organs, flower heads had high values of cichoric acids with 32.39 and 56.15 mg/100 g in *E. purpurea* and *E. pallida*, respectively, while they were 4.96 and 0.74 in leaves of *E. purpurea* and *E. pallida*, respectively. From previous studies, it was determined that flower heads of *Echinacea* had more total phenolic and cichoric acids than leaves (Chen et al. 2009). Major morphological variation in chlorogenic acid with 13.12 and 1.06 was found in flowers of *E. pallida* and *E. purpurea*, respectively.

Diversity in the chemical contents has been ascribed to many factors, involving the environment, abiotic stress, genetic heritance and the developmental stages of the plants.

### TABLE 1. PHENOL COMPOSITION OF *ECHINACEA* SPECIES (LEAF AND FLOWER) EXTRACTED WITH METHANOL AND WATER

<table>
<thead>
<tr>
<th>Composition (mg/100 g)</th>
<th>Methanol extracts</th>
<th>Water extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Echinacea purpurea</em></td>
<td><em>Echinacea pallida</em></td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>Flower</td>
</tr>
<tr>
<td>4-Hydroxy benzoic acid</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>nd</td>
<td>0.01</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>nd</td>
<td>0.39</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>nd</td>
<td>0.03</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>nd</td>
<td>1.06</td>
</tr>
<tr>
<td>Cichoric acid</td>
<td>4.96</td>
<td>32.39</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.01</td>
<td>0.08</td>
</tr>
<tr>
<td>Rutin</td>
<td>0.14</td>
<td>3.36</td>
</tr>
</tbody>
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*nd*, not detected.
FIG. 2. LC-TOF/MS SPECTRUM OF (A) EPA LEAF (H2O), (B) EPU FLOWER (MEOH), (C) EPU FLOWER (H2O)

FIG. 3. LC-TOF/MS SPECTRUM OF (A) EPU LEAF (MEOH), (B) EPU LEAF (H2O), (C) EPA FLOWER (MEOH)
(Yesil-Celiktas et al. 2007; Singh and Guleria 2013). The variation in the chemical constituents has been observed previously in *E. purpurea*. The range of cichoric acid in root changed from 1.7 to 2.3% in different seasons (Perry et al. 2001), and from 0.5 to 0.8% by different plant age or population (Binns et al. 2002). In this work, variation of cichoric acid was also observed between the leaf and flower (Table 1). Rutin, a pharmaceutically and medicinally valuable compound, existed in methanol and water extracts of both plant species. Recent work about the rutin indicated that it has antioxidant (Das et al. 2015), antibacterial (Ananth et al. 2015), antidiabetic (Wang et al. 2015), anticancer (Arinc et al. 2015), antiepileptic (Dubey et al. 2015), antimalarial (Choudhary et al. 2014) and antithyroid (Panda and Kar 2014) properties. The water extract of *E. purpurea* leaves, *E. purpurea* flowers, *E. pallida* leaves and *E. pallida* flowers revealed the excellent ABTS cation radical (Fig. 4), DPPH free radical scavenging activities (Fig. 5) as well as reducing power (Fig. 6). This could not be attributed mainly to the caffeic acid, which is the chief constituent of water extracts of both plant leaf and flower, but also to secondary metabolites that the extracts contained. Methanol extract of *E. pallida* flowers consisted of the highest phenolic contents (Fig. 7). Caffeic acid derivatives, bioactive compounds, were present in *Echinacea* species, particularly *E. purpurea* and *E. pallida*. Caffeic acid (3,4-dihydroxycinnamic acid), a natural phenolic compound, has diversity of biological properties such as anticancer (Chen et al. 2014; He et al. 2014; Yang et al. 2014a), anti-inflammatory (Yang et al. 2014b), antiviral (Utsunomiya et al. 2014).
et al. 2014) and antioxidant (Cheng et al. 2014; Fernandes et al. 2014) activities.

CONCLUSIONS

E. pallida and E. purpurea exhibit excellent antioxidant activities and they contain pharmaceutically and medicinally valuable compounds. Therefore, these species could be used in food and pharmaceutics industries.

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