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Research Article

HPLC PROFILES OF PHENOLIC COMPOUNDS AND ANTIOXIDANT ACTIVITY OF *ECHIUM VULGARE* GROWING IN TURKEY

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ABSTRACT

Echiumvulgare known as viper's bugloss is a species of flowering plant in the borage family Boraginaceae. *Echiumvulgare* was investigated in terms of phenoliccontents and antioxid ant capacity in present study. Antioxidant activity of methanolic extract was examined by two methods, namely ferric reducing antioxidant power (FRAP), and 2,2-diphenylpicrylhydrazyl radical scavenging (DPPH)activity. The phenolic compounds were determined by reverse phase-high performanceliquid chromatography (RP-HPLC). Benzoicacid, chlorogenicacid, caffeicacid, and gallic acid were determined as main phenolic compounds in the methanolic extract. The IC_{50}valuefor DPPH assay has been found as 0.1430±0.0014 (mg/mL), FRAP value is 795±14.1892 (µMTrolox/g sample), and total phenoliccontent value is 10.5±0.1732 mg gallicacidper gram sample in methanolic extract of the aerial parts of *Echiumvulgare*. Themethanolicextract of *Echiumvulgare* containsrichphenolic compounds, and potent antioxidant activity that might be used as raw material by pharmaceutical and food industries for the preparation of natural drugs.

INTRODUCTION

The genus *Echium* (Boraginaceae) is made up of nine species in the flora of Turkey (Edmondson, 1978). The roots of *Echiumvulgare* L. are employed in traditional folk medicine to treat cracked hands and for wound healing (Sezik *et al,* 1997). It has also been described as a major wild honey plant (Klemow *et al,* 2002). Various parts of *Echium* species (including herbs, petals, roots and root barks) have been employed to treat rheumatic pain and for their wound healing, demulcent, diuretic, sedative and antioxidant properties (Ghorbani, 2005; De Natale *et al,* 2007; Niciforovic *et al,* 2010; Mirdeilami *et al,* 2011). The roots of *Echiumvulgare* are reported to be applied externally to treat wounds in traditional Turkish folk medicine (Altundag *et al,* 2011; Yesilada *et al,* 1995). Scientific research has reported antibacterial, anti-inflammatory, antiproliferative, antidepressant, antioxidant, antiviral, anxiolytic and cytotoxic activities in various species of *Echium* (Abed *et al,* 2012; Moallem *et al,* 2007; Farahani *et al,* 2013; Omar *et al,* 2008; Hosseinzadeh *et al,* 2012).

Oxidative stress is implicated as a factor in a wide range of acute and chronic diseases, such as cancer, cardiovascular disorders and neurodegenerative conditions. The balance between antioxidation and oxidation must be maintained if a biological system is to be preserved in a healthy state (Hong *et al,* 2004; Katalinic *et al,* 2006). Medicinal plants contain high levels of natural antioxidants, such as phenolic acids, flavonoids and tannins, exhibiting potent antioxidant activities (Wong *et al,* 2006; Rammal *et al,* 2013).

Weinvestigated the total phenolic content, antioxidant activity of *Echiumvulgare*, and also determined phenolic compounds by using RP-HPLC.

MATERIALS AND METHODS

Chemicals and Instrumentation

The phenolic standards were of HPLC grade. Gallic acid, protocathechuic acid, proto-catechuic aldehyde, *p*-hydroxybenzoic acid, chlorogenic acid, vanillic acid, caffeic acid, vanillin, syring aldehyde, *p*-coumaric acid, ferulic acid, sinapic acid, and benzoic acid were purchased from Sigma-Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany). Acetic acid, acetonitrile, methanol, trolox (6-hydroxy–2,5,7,8-tetramethylchroman–2-carboxylic acid),

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TPTZ (2,4,6-tripyridyl-s-triazine), and folin-Ciocalteu's phenol reagent were obtained purchased from Merck (Darmstadt, Germany) and FlukaChemie GmbH (Buchs, Switzerland). Polytetrafluoroethylene membranes (porosity 0.45 µm) for extract filtration were purchased from Sartorius (Goettingen, Germany).

Phenolic compounds were analyzed using high performance liquid chromatography (HPLC) (Agilent 1100, DAD 1200 Agilent Technologies, Waldbronn, Germany) in this study. The experimental protocol included a reverse phase waters spherisorp ODS2-C18 column (4.6×250 mm, 5 µm) on a gradient program with a two-solvents system (A: 2% acetic acid in water; B: 0.5% acetic acid in acetonitrile: water [1:1]) at a constant solvent flow rate of 1.2 mL.min-1. The injection volume was adjusted to 20 µL. Signals were identified at 232, 246, 260, 272, 280, 290, 308 and 328nm by DAD and at 280 nm through UV detection. The column was kept at a steady room temperature of 25°C.

All absorbance measurements were performed on a Spectro UV-Vis Double PC–8 auto cell spectrophotometer (Labomed Inc., California, U.S.A.). Solutions were made ready with deionized water purified in an Elgacan® C114 Ultra Pure Water System Deionizer device (The Elga Group, Buckinghamshire, England).

Evaporation was performed with an IKA® RV 05 Basic (IKA®, Werke, USA) rotary evaporator system, and extraction using a HeidolphPromax 2020 (Heidolph Instruments GmbH & Co., Schwabach, Germany) shaker. A HeidolphReax top vortex (Heidolph Instruments GmbH & Co., Schwabach, Germany) and Elma® Transsonic Digital ultrasonic water bath (Singen/Htw, Germany) were used during all dissolution procedures. pH measurements were performed with the assistance of a Hanna (HI 110 series) instruments microprocessor pH meter (Hanna Instruments, Inc., Rhode Island, U.S.A.), and a Mettler Toledo (Mettler-Toledo GmbH., Gießen, Germany) scale was used for all weight calculations, expressed in grams.

Preparation of extract for phenolics and antioxidant analysis

Echiumvulgare was gathered from Yusufeli, Artvin in spring between May and June, 2014, and biological authentication was carried out by Professor UfukOzgen. The dried powder obtained from aerial parts of *Echiumvulgare* (1 g) was extracted with 20 mL methanol in a flask attached to the condenser in a sonicator device at 60 ºC over 3 h.Antioxidant activities were determined by removing 10 mL from each extract. The residual methanol extracts were then evaporated to dryness before being concentrated in a rotary evaporator at 50 ºC. The crude extract was subsequently dissolved in a total of 10 mL distilled water, following which liquid-liquid extractions were carried out. The extraction procedure was carried out three times with 5 mL diethyl ether and 5 mL ethyl acetate. Once the organic moiety had been picked up in the same flask, this was evaporated to dryness under decreased pressure with the help of a rotary evaporator at 40 °C. In the final stage, the residue was weighed and dissolved in methanol for HPLC analysis.

Determination of Antioxidant Capacity

Total phenolic contents (TPC) were identified using the procedure described by Folin-Ciocalteau with gallic acid as standard. Briefly, 0.1 mL of various concentrations of gallic acid and methanolic samples (1 mg.mL-1) were diluted using 5.0 mL distilled water. In the following step, 0.5 mL of 0.2 N Folin-Ciocalteureagent was added and subjected to vortexing. Following 3-min incubation, 1.5 mL of Na2CO3 (2%) solution was added. The mixture was then incubated with occasional shaking for 2 h at 20 0C. Absorbance was measured at 760 nm once the incubation period had concluded. TPC concentrations were calculated in the form mg of gallic acid equivalents per gram of 100 g sample, with the help of a standard graph.

The ferric-reducing/antioxidant power (FRAP) assay, based on the measurement of ferric reducing capacity, was used to calculate antioxidant activities in methanolic specimens. We employed the FRAP assay technique previously described by Benzie and Straine (1996), with a number of minor modifications. Working FRAP reagent was prepared as required by mixing 25 mL of 0.3 M acetate buffer at pH 3.6 with 2.5 mL of 10 mM 2,4,6-tripyridyl-S-triazine (TPTZ) solution in 40 mMHCl and 2.5 mL of 20 mM FeCl3.6H2O. In the next stage, 100 µL of sample was combined with 3 mL of fresh FRAP reagent. This was subsequently incubated at 37 0C for 4 min. Absorbance was calculated at 593 nm against a blank produced with distilled water and incubated for 1 h, instead of 4 min. A calibration curve was employed, using Trolox concentrations in the range of 100–1000 µM, $r2$=0.97. For purposes of comparison, Trolox® was also tested under identical conditions in the form of a standard antioxidant compound. FRAP values were expressed as µMTrolox equivalent of g sample.

Butylated hydroxytoluene (BHT) was adopted as a reference antioxidant compound in the course of DPPH assay (Molyneux 2004). The differing concentrations of 0.75 mL of methanolic extract of *E. vulgare* were combined with 0.75 mL of 0.1 mM of DPPH in methanol. Radical scavenging activities were expressed as IC50 (mg sample per mL), a value that represents the concentration of samples resulting in 50% scavenging of DPPH radicals.

RESULTS AND DISCUSSION

Both the general public and medical professionals have shown increasing interest in the use of indigenous drugs for therapeutic purposes in recent years (Siddique *et al,* 2010). Plant foods (fruits, grains and vegetables) are the subject of particular interest since these contain nutrients and bioactive components (phytochemicals). Polyphenols are micronutrients present at significant levels in the human diet and also found in numerous medicinal plants (Manach *et al,* 2004). The antioxidative activity of phenolics contained in functional foods derives from direct free radical scavenging activity and reducing activity and from an indirect effect caused by the chelation of metal ions (Mustafa *et al,* 2010; de Oliveira *et al,* 2009). The potential therapeutic attributes of numerous traditional medicinal plants may therefore largely be attributed to the phenolic compounds contained within them (Aliyazicioglu *et al,* 2015). Someauthors (Stanojevic *et al*, 2009; Siddique *et al*, 2010; Aliyazicioglu *et al,* 2013) have

demonstrated a positive correlation between the content of phenolic compounds and their antioxidant activity. Differences in levels of antioxidant activity may therefore be associated with the nature of the phenolic compounds involved, from phenolic acids to flavonoids, rather than with their contents (Kahkonen *et al,* 2009; Mhamdi *et al,* 2010). For example, the radical scavenging properties of phenolic acids and their derivatives, such as esters, as well as flavonoids in plants may derive from the number of hydroxyl groups the molecules contain (Soobrattee *et al*, 2005; Siddique *et al,* 2010). Some extracts exhibit high phenol/flavonoid concentrations but low antioxidant activities. Others, in contrast, exhibit low phenol/flavonoid concentrations but high antioxidant activities. The varying different reactions of phenolic compounds to the Folin–Ciocalteu reagent derive from the numbers of their phenolic hydroxyl groups (Wong *et al,* 2006).

Total phenolic content was determined from the calibration curves of gallicacid. TPC value of *Echiumvulgare* extract was calculated at 10.5 ± 0.1732 mg GAE/100 g dry weight (DW) (Table 1). Eruygur*et al*. (2012) reported TPC at a level of 9.71 ± 0.03 mg GAE/g for herbs and 16.82 ± 0.01 mg GAE/g for roots of *Echiumvulgare*. Abbaszadeh *et al,* (2013) reported that the TPC values of the leaf extracts ranged from 17.42 ± 0.47 mg GAE/g DW in *E. italicum*to119.50 ± 2.00 mg GAE/g DW in *E. amoenum*.

Table 1 The antioxidant activities of the methanolic extract (MeOH) of *Echiumvulgare.*

Test compounds	TPC1	FRAP2	DPPH3
MeOH	10.5± 0.1732	795±14.1892	0,1430±0,0014
BHT			0.0099 ± 0.0002

1Total phenolic content expressed in mg of gallicacid equivalent (GAE) per gram of dry plant weight, 2Expressed as µMtrolox equivalents (TE) per gram of dry plant weight, 3Concentration of test sample (mg/mL)required to produce 50 % inhibition of the DPPH radical.

A range of biologically active elements in plants provide protection against various physical and chemical hazards, such as diseases, parasites and bacteria (Kolayli *et al,* 2010; Aliyazicioglu *et al,* 2013). Their phenolic contents also permit them to exhibit bioactive properties. Samples will invariably possess a spectrum of various phenolic compounds, and individual measurement of these may consequently be problematic. RP-HPLC was used to analyze 13 phenolic acids such asgallic acid, proto-catechuic acid, proto-catechuic aldehyde, p-hydroxy benzoic acid, chlorogenic acid, vanillic acid, caffeic acid, vanillin, syringaldehyde, *p*-coumaric acid, ferulic acid, sinapic acid, benzoic acid. The RP-HPLC chromatograms elicited from the standard phenolic compounds in this study are shown in Fig. 1.

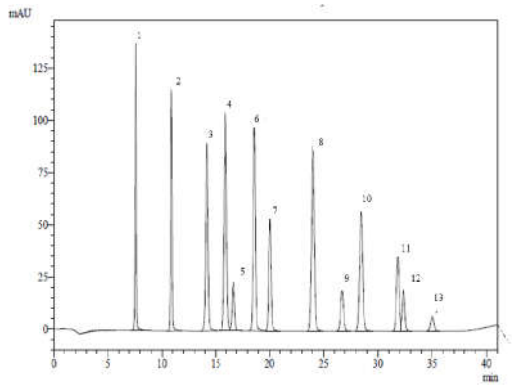

Figure1

Figure1 RP-HPLC chromatogram of phenolic standards (25 µM) searched in *Echiumvulgare*samples. Waters spherisorp ODS2 -C18 column (4.6×250 mm, 5 µm), gradient eluent acetic acid/acetonitrile/water, flow rate 1.2 mL/min. Peak identification: (1) gallic acid, (2) *proto*-catechuic acid, (3) *proto*-catechuic aldehyde, (4) *p*-OH benzoic acid, (5) chlorogenic acid, (6) vanillic acid, (7) caffeic acid, (8) vanillin, (9) syring aldehyde, (10) *p*-coumaric acid, (11) ferulic acid, (12) sinapic acid, (13) benzoic acid.

Comparison of the individual phenolic compounds contents identified benzoic acid as the main phenolic component in aerial parts of *Echiumvulgare*. Also, benzoic acid, chlorogenicacid, caffeicacid, and gallicacidwas identified from aerial parts of *Echiumvulgare*. (Fig. 2 and Table 2).

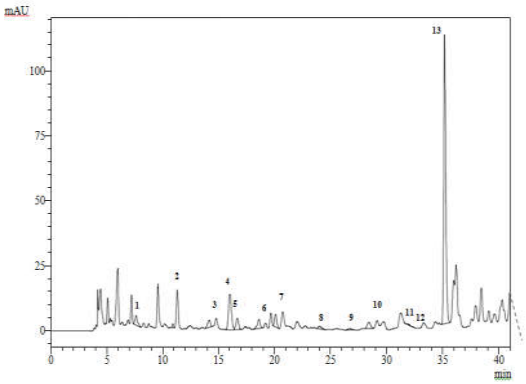

Figure 2

Figure 2 RP-HPLC DAD chromatogram of *Echiumvulgare* pH 7 methanol extract (50 mg/mL) at 280 nm. Waters spherisorp ODS2 -C18 column (4.6×250 mm, 5 µm), gradient eluent acetic acid/acetonitrile/water, flow rate 1.2 mL/min. Peak identification: (1) gallic acid, (2) *proto*-catechuic acid, (3) *proto*-catechuic aldehyde, (4) *p*-OH benzoic acid, (5) chlorogenic acid, (6)vanillic acid, (7) caffeic acid, (8) vanillin, (9) syring aldehyde, (10) *p*-coumaric acid, (11) ferulic acid, (12)sinapic acid, (13) benzoic acid.

The FRAP method was employed to identify antioxidant capacities in this study. This involved sample antioxidants reducing the ferric tripyridyltriazine (Fe-(III)-TPTZ) complex to a blue-colored Fe (II)-TPTZ. The reduction of ferric iron in FRAP reagent gave a blue product (ferrous–TPTZ complex), the absorbance of which can be read at 593 nm in the samples, with those containing Trolox at a known concentration of 1,000 µM.

Table2 Phenoliccomposition of the methanolic extract of *Echiumvulgare*

Phenolic compound assignment	Retention time (min)	Amount (mg/L)
Gallicacid	7.319	1.614
Protocatechuicacid	10.506	0.686
Protocatechuicaldehyde	14.069	1.306
p-OH-benzoicacid	15.584	3.926
Chlorogenicacid	16.720	5.382
Vanillicacid	18.557	1.374
Caffeicacid	19.900	2.458
Vanillin	24.020	0.757
Syringaldehyde	26.646	1.252
p-Coumaricacid	27.949	1.401
Ferulicacid	31.682	0.619
Sinapicacid	32.531	0.544
Benzoicacid	35.361	225.357

Greater absorbance in this assay indicates a greater reducing capacity. Since the total reducing power represents the sum of the reducing powers of the individual compounds within a sample, the FRAP test is regarded as a goodindicator of total antioxidant power. FRAP value of *Echiumvulgare* extract was calculated at 795± 4.1892 (µMTrolox/g sample) (Table 1). Abbaszadeh *et al.* (2013) reported that the highest FRAP values were for seed extracts of *E. amoenum* obtained from the Behshahr (20.88±0.72 mg GAE/g DW) and Ramsar (20.66±0.47 mg GAE/g DW) locations. Seed extract of *E. italicum* (Kaleybar) exhibited the lowest FRAP value, at 4.90±0.13 mg GAE/g DW. The FRAP values of leaf extracts ranged from 0.78 ± 0.00 to 1.95±0.09 mg GAE/g DW for *E. amoenum* collected from Behshahr and Ramsar, respectively.

The free radical scavenging effect of *E. vulgare* extract was determined using the DPPH test with BHT as a reference. The highest level of DPPH radical scavenging power was identified in the methanolic extract of the plant. This may be attributed to significant levels of variation in the polyphenols in the samples, in line with their scavenging capacities. It may be hypothesized that the phenolic compounds acted as free radical scavengers due to their hydrogen-donating characteristic (Molyneux, 2004). The methanolic extract exhibited hydroxyl radical scavenging properties acting as a hydrogen atom or electron donor during the DPPH test. This improved iron autoxidation, while causing a marked decrease in the accessibility of iron to oxygen molecules by oxidizing ferrous ion to a ferric state, a phenomenon which subsequently inhibited hydroxyl radical production (Yoshino *et al,* 1998). These results strongly suggest a relationship between the antioxidant activity of the methanolic extract and a high level of phenolic compounds. Inthisstudy, the IC50valuefor DPPH assay has been found as 0.1430 ± 0.0014 (mg/mL) in methanolic extract of the aerial parts of *Echiumvulgare*. Eruygur *et al*, (2012) reported that among the extracts, *E. italicum* root extract showed high scavenging activity with 81.43±0.01%, followed by *E. angustifolium* and *E. Vulgare* root extract with 75.49±0.11% and 71.20±0.01 % and *E. Parviflorum* herb extract with 68.84±0.03 % in 1000 µg/ml concentration.

CONCLUSIONS

The results of studied parameters clearly indicate that the extract of *Echiumvulgare* have rich phenolic compositions, and antioxidant activity due to its numerous different secondary metabolites, polyphenol in particular. *Echiumvulgare* may beuse as raw material by the pharmaceutical, and food industries to prevent several oxidative stress-related diseases such as cardiovascular and aging-associated diseases by neutralizing free radicals in the body.

Abbreviations Used

FRAP, ferric-reducing/antioxidant power; DW, dry weight; GAE, gallic acid equivalents; RP-HPLC, reverse phase-high performance liquid chromatography; Trolox®, 6- hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; TPTZ, 2,4,6-tripyridyl-s–triazine; BHT, butylatedhydroxytoluene; DPPH, 2,2-diphenyl-1-picrylhydrazyl.

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