Research Paper

Phenolic Composition, Antioxidant and Cytotoxic Activities of *Prospero autumnale*

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Plants of the *Prospero* genus have been used for promoting blood circulation, treating nervous conditions, infertility in women and rheumatic fever in the traditional folk medicine. The aim of present research was to investigate the phenolic composition, antioxidant and cytotoxic activities of the aerial and underground parts of *Prospero autumnale*. While β-carotene/linoleic acid, metal chelating and phosphomolybdenum assays were used for the determining antioxidant activity, Folin-Ciocalteu assay, aluminium colorimetric and vanillin-sulphuric acid method were used to detect total phenolic, flavonoid and saponin contents in the extracts, respectively. The phenolic compounds of *P. autumnale* were estimated by high performance liquid chromatography analysis. Nine phenolics were identified in the extract. Quercetin was the major constituent in the extract and is mainly responsible for biological activities observed. Aerial parts of the plant showed higher antioxidant activity than the underground parts and this activity may be related to a good total phenolic content. *In vitro* cytotoxic activity was determined by a luminometric method against non-small cell lung carcinoma cells. Underground parts of the plant exhibited more than fifty percent mortality on H1299 cells at a concentration of 500 µg/ml. This cytotoxicity could be due to the saponins, which have been suggested as possible anticarcinogens in plants. Therefore, further in depth studies are needed elucidate the mechanism involved in cytotoxic activity of *P. autumnale* extract along with isolation and identification of active principles.

Key words: Antioxidant activity, *Prospero autumnale*, cytotoxicity, HPLC

Nature has been a source of traditional or modern medicinal products for thousands of years. Since ancient times, plants have been used by humans for treatment of various diseases and a great number of active ingredients of modern drugs have been isolated from natural sources. Especially, the plant-based, traditional medicine systems have become popular again because of the adverse effects of synthetic drugs[1].

Geophyte plants are those, which develop underground parts such as tubers, bulbs and rhizomes specifically to store nutrients. Eight hundred and sixteen geophyte species belonging to 73 genera have been registered in Flora of Turkey[2]. *Scilla autumnalis* L. is a synonym of *Prospero autumnale* (L.) Speta, an autumnal flowering plant of the family Asparagaceae, is found in the Mediterranean region from Portugal to Turkey and the Caucasus[3].

There has been no report on the possible medical uses of *P. autumnale* (as *S. autumnalis*) in the literature but it is known that *Scilla* species are widely used in folk medicine to treat different illness related to inflammation and pain. Phytochemical studies have demonstrated the presence of triterpene and triterpenoid saponins derived from alkaloids, eucosterol, stilbenoids and lignan in the plants of this genus. In relation to these substances, several bioactivities such as antioxidant, antitumor, antiinflammatory, cardioprotective and glycosidase inhibitory activities, have been reported[4-6]. *S. scilloides* has been used for a long time by traditional Chinese healers to treat abscesses and to promote circulation. The bulb extract has been evaluated for its potential as an antimicrobial agent, as an antiinflammatory and

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as an antioxidant[7]. Some genera of the Asparagaceae have been investigated, with the bulbs receiving most attention. Investigations into the chemistry of the bulbs of *Scilla* has yielded a large number of nortriterpenoids, cardiac glycosides and homoisoflavanones, many of which have been evaluated for biological activity[8-10].

While most of the studies represent the biological activities of underground parts (bulbs) of the *P. autumnale*, there are few studies about the aerial parts of this plant. In the light of all information mentioned above, the present study aimed to investigate the potential antioxidant and cytotoxic activities of aerial and underground parts of the ethanolic extract of *P. autumnale.* The phenolic compounds of aerial parts of the plant were also detected by reversed-phase high performance liquid chromatography (RP-HPLC) in this study.

MATERIALS AND METHODS

P. autumnale was collected in October 2015 from Denizli, Turkey and authenticated in the Department of Biology, Pamukkale University, Denizli, Turkey. A voucher specimen (RM1002) has been deposited in the Herbarium of the Department of Biology. Aerial and underground parts of the plant were air-dried in shade at 25°, powdered to a fine grain and then extracted with 100 ml ethanol at 50° for 6 h in temperature controlled shaker[11]. The ethanol extract was filtered with Whatman filter paper (No. 1) and evaporated to remove the ethanol or dryness under vacuum at temperature below 50° using the rotary evaporator (IKA RV10D, Staufen, Germany). A minimum volume of distilled water was added to the dry ethanol extract and the resulting material was freeze-dried (Labconco FreeZone, Kansas City, MO). Obtained extracts were stored at –20° until use.

β-carotene/linoleic acid assay:

β-carotene/linoleic acid assay was carried out by the method reported by Sokmen *et al*.[12]. A stock solution of β-carotene-linoleic acid mixture was prepared by dissolving 0.5 mg β-carotene in 1 ml chloroform. About 25 µl linoleic acid and 200 mg Tween 40 were added. Chloroform was completely evaporated using a vacuum evaporator. Then 100 ml of distilled water was added with vigorous shaking. Also, 2.5 ml of this reaction mixture was dispensed into test tubes and 350 µl portion (1 mg/ml) of the extract was added and the emulsion system was incubated for up 2 h at 50°. The same process was done again with synthetic antioxidant, butylated hydroxytoluene (BHT), as positive control, and a blank. The absorbance of the mixtures was measured at 490 nm after the incubation period, and inhibition ratio was calculated.

Total antioxidant capacity:

The total antioxidant capacity of extract was assessed by phosphomolybdenum method according to Prieto *et al*.[13]. About 0.3 ml of extract solution (1 mg/ml) was mixed with 3 ml of reagent solution (6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The reaction mixture was incubated for 90 min at 95°. Then, the absorbance of the solution was measured at 695 nm against blank. The antioxidant capacity of extract was expressed as equivalents of ascorbic acid (mg AEs/g).

Metal chelating activity on ferrous ions:

The metal chelating activity on ferrous (Fe^{+2}) ions of the extract was estimated by the method previously reported by Aktumsek *et al*.[14]. Shortly, sample solution (2 ml) was added to $FeCl_2$ solution (0.05 ml, 2 mM). The reaction was started immediately by adding 5 mM of ferrozine (0.2 ml). In the same way, a blank was prepared by adding sample solution (2 ml) to $FeCl_2$ solution (0.05 ml, 2 mM) and water (0.2 ml) without ferrozine. Then, the sample and blank were left at room temperature for 10 min and the absorbance's were measured at 562 nm. The metal chelating activity was expressed as equivalents of EDTA (mg EDTAs/g).

Total phenolic content:

Total phenolic content of the extract was determined by Folin-Ciocalteu method[15] with a slight modification. About 1 ml of extract solution (1 mg) was added to 46 ml of distilled water and 1 ml of Folin-Ciocalteu reagent and was mixed properly. After 3 min, the mixture was added to 3 ml of sodium carbonate (2%) and shaken intermittently for 2 h. The absorbance was measured at 760 nm. Gallic acid was used as a standard for calibration curve. The total phenolic content was expressed as gallic acid equivalents (mg GAEs/g).

Total flavonoids content:

Total flavonoids content of the extract was determined by aluminium colorimetric method according to Arvouet-Grand, *et al*.[16]. Briefly, 2000 µg (1 ml) extract was mixed with 1 ml of 2% aluminium trichloride ($AlCl_3$) in methanol. Similarly, a blank was prepared by adding extract solution (1 ml) to methanol (1 ml) without $AlCl_3$. After 10 min incubation at room

temperature, the blank and extract absorbance's were measured at 415 nm. The total flavonoids content was expressed as quercetin equivalents (mg QEs/g).

Total saponin content:

Total saponin content was determined by the vanillin-sulphuric acid method. The extracts were mixed with the same amount of vanillin (8%, w/v) and twice the amount of sulphuric acid (72%, w/v). The mixture was incubated at 60° for 10 min followed by cooling in an ice water bath for 15 min. Absorbance was measured at 535 nm. The total saponin content was expressed as equivalents of Quillaja (mg QAEs/g)[17].

Quantification of phenolic components by RP-HPLC:

Phenolic components were evaluated by RP-HPLC. Detection and quantification were carried out with a LC-20AT pump, a Diode Array Detector (SPD-M20A), a CTO-10ASVp column heater, SCL-10Avp system controller, DGU-14A degasser and SIL-10ACHT auto sampler. Separations were conducted at 30° on C-18 reversed-phase column (250×4.6 mm length, 5 µm particle size). The eluates were identified at 278 nm. The mobile phases were 3.0% formic acid in distilled water and methanol. Ethanol was used to dissolve samples, and then 20 µl of this solution was injected into the column. Phenolic composition of the extract was determined according to the method of Caponio *et al*.[18] with a slight modification. Kaempferol, quercetin, (-)- epicatechin, myricetin, quercetin-3-O-glucoside, caffeic acid, rutin, (+)- catechin, sinapic acid, ferulic acid, chlorogenic acid, gallic acid and vanillic acid (Sigma Chemical Co.) were used as standard. The differentiation and quantitative analysis were made by comparing the standards. The quantity of each phenolic compound was expressed as mg/g of the extract.

Cytotoxicity assay:

H1299 cells were cultured in RPMI 1640 medium (Sigma Aldrich, St. Louis, MO, USA) at 37° in a CO_2 incubator. When the cells were grown to about 90% confluence, the medium was aspirated. Cells were washed, trypsinized, counted with a hemocytometer, and seeded into 96-well plates (2×10^3 cells/well). After 24 h incubation, the medium was removed from the well leaving the adherent cells and cells were treated with plant extract in different concentrations (1000, 500, 250, 100, 50, 10 and 1 µg/ml) for 72 h. For the untreated control group, cells were not treated with any extracts. At the end of incubation time, medium was removed, and cytotoxicity of plant extract-treated and untreated control groups was determined by the luminometric method using a CytotoxGlo kit[22].

RESULTS AND DISCUSSION

Antioxidant activities of the aerial and underground parts of *P. autumnale* were analysed by using β-carotene/linoleic acid, metal chelating and phosphomolybdenum assays. β-carotene/linoleic acid bleaching test analysed by the power to neutralize the free radicals formed in the system, which attack the highly unsaturated β-carotene models[19] and these results are shown in Table 1. Aerial parts of the plant demonstrated higher antioxidant activity than underground parts with the inhibition value of 70.34% and 65.71%, respectively, which indicated the role of structural features of polyphenolic compounds with respect to their antioxidant potential. Inhibition rate of oxidation of linoleic acid of *S. maritima* bulbs was reported as 19.77%, which was lower than *P. autumnale*[20]. A previous study reported that ethanol extract of the bulbs of *S. scilloides* exhibited significant antioxidant activity. Antioxidant activity was assessed by monitoring the oxidation of linoleic acid and good activity was found (antioxidative index of 33.2 at a concentration of 1%)[7]. Data obtained from the phosphomolybdenum assay was found in correlation with those obtained from total phenolic assay. In phosphomolybdenum assay, aerial parts of the plant (55.22 mg AEs/g), showed higher antioxidant activity than the underground parts (47.15 mg AEs/g) (Table 2).

While natural bioactive compounds such as plant phenolics could reverse the pathological conditions, reactive oxygen species cause harmful effects leading to several disorders[21]. Total phenolics and flavonoids content were found to be maximum (16.03 mg GAEs/g, 25.01 mg QEs/g) in aerial part of the plant in relation to its antioxidant activity (Table 2). In a previous study, *Scilla hanburyi* was evaluated for the total phenolic

TABLE 1: ANTIOXIDANT ACTIVITIES OF *P. AUTUMNALE*

Sample	β-carotene/ linoleic acid assay (%)	Metal chelating activity (mg EDTAEs/g)[a]
Aerial part	70.34±0.04[b]	7.14±0.11
Underground part	65.71±0.03	5.03±0.07
BHA	92.04±0.10	nt
BHT	93.02±0.21	nt

[a]EDTAEs: EDTA equivalents, nt: no tested. [b]Values expressed are means ±SD

constituents. Total phenolic contents of the aqueous and methanolic extracts of *S. hanburyi* were detected as 17.3 and 6.9 mg GAEs/g, respectively. The results of total phenolic contents demonstrated a uniform tendency to those of the antioxidant capacities. For this reason, the high content of total phenolics in the extract might be state the strong antioxidant capacity of *P. autumnale*. These results are in good agreement with previously published reports in the literature, which exhibited strong connection between antioxidant activities and total phenolic contents[22,23].

Plant extracts were also assessed for their metal chelating activities, which are linked to antioxidant capabilities. Metal chelating assay was based on the measurement of iron-ferrozine absorbance at 562 nm. The chelating activities of the extracts were established using EDTA as a standard (mg EDTAEs/g extract). In good agreement with results of other antioxidant assays, potent chelation capacities were again detected in the aerial parts of the plant with 7.14 mg EDTAEs/g (Table 1). Tripathi *et al*.[24] examined the antioxidant capacity of the bulb of *S. indica* in the way of its effect on ferrous sulphate-induced lipid peroxidation and concentration of reduced glutathione in mice liver homogenate and on hydroxyl radicals, superoxide radical scavenging and iron chelation in a chemical model. The results exhibited that the ethanolic bulb extract of *S. indica* inhibited lipid peroxidation and chelated transition metals in the body.

Phenolic components are a diverse group of phytochemicals widely distributed in the plant kingdom[25], therefore the phenolic components contained in the aerial parts of the *P. autumnale* ethanolic extract were characterized using HPLC. Out of the 13 standard phenolics analysed, 9 phenolic components were identified in the extract and listed in Table 3. Also, HPLC chromatograms of phenolic components in the *P. autumnale* were shown in fig. 1. Major phenolic components in the extract were determined as quercetin (2.33 mg/g extract) and caffeic acid (2.15 mg/g extract). These data indicated that the antioxidant activities of *P. autumnale* could be ascribed to their polyphenol components. Quercetin is considered to be a strong antioxidant due to its ability to scavenge free radicals and bind transition metal ions[26]. Therefore, high concentration of quercetin was thought to be responsible for the antioxidant activities performed. As a result, quercetin may aid in the prevention of certain diseases, such as cancer, atherosclerosis, and chronic inflammation[27]. A previous study reported that eighteen polyphenols were identified from extracts of the aerial and underground parts of *S. bifolia* (caftaric acid, isoquercitin, routine, myricetol, fistein, quercetol, patuletin, gentisic acid, caffeic acid, chlorogenic acid, *p*-coumaric acid, ferulic acid, hyperoside, quercitrin, luteolin, kaempferol, apigenin and sinapic acid)[28].

TABLE 2: TOTAL ANTIOXIDANT CAPACITY, TOTAL PHENOLIC AND FLAVONOID CONTENTS OF *P. AUTUMNALE*

Material	TPC[a]	TFC[b]	TAC[c]
Aerial part	16.03±0.05[d]	25.01±0.08	55.22±0.09
Underground part	10.01±0.04	15.34±0.02	47.15±0.08

[a]Total phenolic content (TPC) expressed as gallic acid equivalents (mg GAEs/g; [b]total flavonoid content (TFC) expressed as quercetin equivalents (mg QEs/g); [c]total antioxidant capacity (TAC) expressed as ascorbic acid equivalents (mg AEs/g); [d]values expressed are means±SD

TABLE 3: PHENOLIC COMPONENTS IN THE AERIAL PARTS OF *P. AUTUMNALE* (MEAN±SD)

Phenolic components	mg/g extract
Kaempferol	1.02±0.10
Quercetin	2.33±0.19
(-)- Epicatechin	0.09±0.06
Myricetin	0.08±0.02
Quercetin-3-O-glucoside	0.02±0.02
Caffeic acid	2.15±0.08
Rutin	0.03±0.02
(+)-Catechin	0.07±0.05
Sinapic acid	0.44±0.07
Ferulic acid	nd[a]
Chlorogenic acid	nd
Gallic acid	nd
Vanillic acid	nd

[a]Values not determined

The extracts were evaluated for cytotoxicity against H1299 human non-small cell lung carcinoma cell line using the luminometric test. The underground parts of *P. autumnale* were found to be more cytotoxic than the aerial parts. Cytotoxicity of the underground parts on the proliferation of H1299 cell line was determined as 56.7% (fig. 2). The cell viability of the extract decreased with increasing the concentration. Saponins are natural glycosides, which possess a wide range of pharmacological properties including cytotoxic activity[29]. The saponin contents of the underground parts of *P. autumnale* extract were found as 148 mg QAEs/g, while the aerial parts of extract were 51 mg QAEs/g. In a previous study, the toxic effects of the aqueous extract obtained from the underground parts of *S. nervosa* in HepG2 liver cells, were investigated[30]. Results indicated that extracts of underground parts had cytotoxic activity on liver

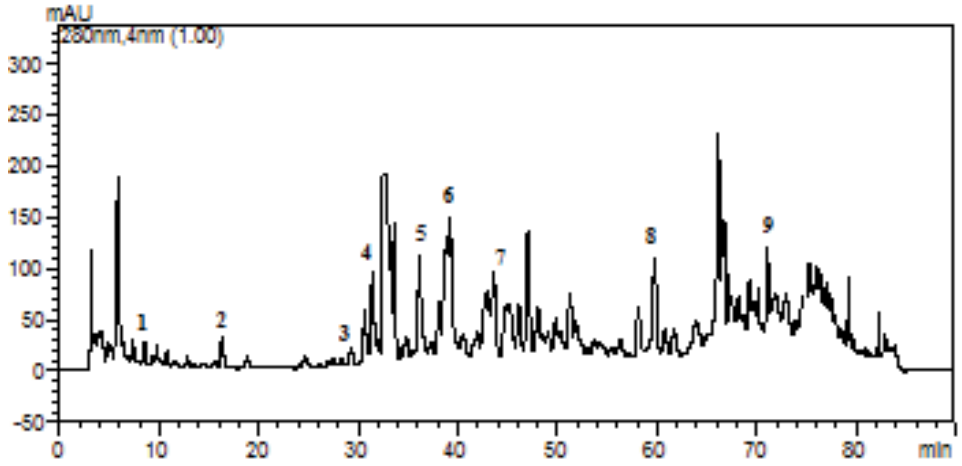

Fig. 1: HPLC chromatogram of phenolic components in the aerial parts of *P. autumnale*
1: Quercetin-3-O-glucoside, 2: (+)- catechin, 3: (-)- epicatechin, 4: myricetin, 5: caffeic acid, 6: rutin, 7: quercetin, 8: sinapic acid, 9: kaempferol

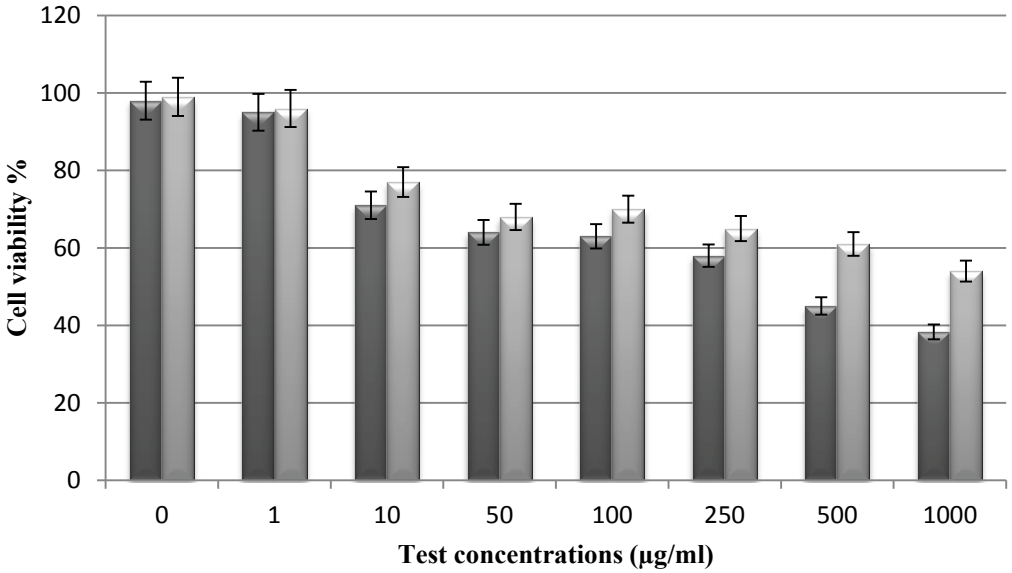

Fig. 2: Cytotoxicity of *P. autumnale* on H1299 cells proliferation
Data are the average results of three independent experiments and expressed as mean±SD. ■ Underground part; ■ aerial part

cells. An ethanolic extract of *S. indica*, has reportedly shown good inhibition of the Semliki Forest Virus and the active principal found to be a diosgenin saponin[31]. Sparg *et al*.[32] investigated anticancer activity of aqueous, ethanolic, dichloromethane and n-hexane extracts of *Scilla natalensis*. The phytochemical screening of underground parts of *S. natalensis* revealed the presence of saponins and bufadienolides within its underground parts[33]. It is tempting to speculate that the observed cytotoxic activity of the underground parts of *P. autumnale* is contributed to the presence of saponins.

Biological activity and chemical composition of *P. autumnale* have been performed for the first time in this study. In terms of the biological activity assays, antioxidant and cytotoxic activities of the extracts were exhibited. Aerial and underground parts of *P. autumnale* extracts possess remarkable biological properties. Traditional uses of some bulbous plant species, mostly belonging to the Asparagaceae, Amaryllidaceae and Hyacinthaceae could provide beneficial leads in novel pharmaceutical progressions. By reason of the pharmacological action and biochemical content of several of this species still remains poorly understood, future investigations should be a valuable aid in this respect.

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Conflicts of interest:

There are no conflicts of interest.

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