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Antioxidant, anti-acetylcholinesterase, and anticancer activities of four *Polygonum* species from Istanbul

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Abstract

Polygonum species are used in traditional medicine in many countries; some are also consumed as vegetables in Turkey. The ethanolic, methanolic, and chloroform extracts of four *Polygonum* species growing in Istanbul, namely *P. aviculare, P. patulum* subsp. *pulchellum, P. lapathifolium*, and the only endemic species *P. istanbulicum* were evaluated for their antioxidant, anti-acetylcholinesterase (AChE), and anticancer potentials. The total phenolic and flavonoid contents of the extracts were determined by Folin-Ciocalteu and aluminium chloride methods, respectively. The antioxidant capacities of the extracts were determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and ferric-reducing antioxidant power (FRAP) assays. The AChE inhibitory activities of the extracts were determined using the Ellman method. Each extract was screened for cytotoxic activity against NRK-52E and HeLa cervical cancer cell lines using the MTT assay. Among the extracts screened, ethanolic extract of *P. istanbulicum* showed the highest total phenolic (207.03 ± 14.12 mg GAE/g extract) and total flavonoid (124.95 ± 7.84 mg CE/g extract) contents, and antioxidant activity (DPPH EC_{50}, 8.09 ± 0.50 mg/mL). The chloroform extract of *P. lapathifolium* exhibited the lowest total phenolic (22.33 ± 3.05 mg GAE/g extract) and total flavonoid (11.66 ± 0.36 mg CE/g extract) contents, and antioxidant activity (DPPH EC_{50}, 218.44 ± 24.46 mg/mL). The extracts exhibited AChE inhibitory activity in a dose-dependent manner, particularly the ethanolic extract of *P. istanbulicum* which displayed strongest inhibition against AChE (88.2 ± 3.44%). AChE inhibition was minimal (32.19 ± 2.09 to 48.34 ± 3.41%) in the chloroform extracts. All ethanolic extracts revealed cytotoxic activity toward HeLa cells, while they were not cytotoxic toward NRK-52E cells. The ethanolic extract of *P. lapathifolium* showed the most potent cytotoxicity against HeLa cells (IC_{50}, 8.70 ± 1.35 µg/mL). Results suggested that ethanol was the best solvent for extracting the phenolic, antioxidant, and anti-AChE compounds, and *P. istanbulicum* may be a potential source of these compounds. Further investigations are nevertheless required to identify the bioactive compounds present in *Polygonum* species.

Introduction

Oxidative stress is described as an imbalance between free radical production and antioxidant protection, and has been linked to the development and progression of many diseases, such as diabetes, heart diseases, neurodegenerative diseases, and cancers (Adwas *et al.,* 2019; Rodrigues *et al.,* 2019). Antioxidants prevent or eliminate diseases caused by oxidative stress by counteracting the harmful effects of free radicals (Neha *et al.,* 2019). Plants contain various antioxidant molecules that prevent oxidation, and act as radical scavengers (Patel *et al.,* 2020). Many plant-derived pharmaceuticals (PDP), mostly developed based on ethnomedicinal knowledge, are used to treat and prevent various diseases (Fabricant and Farnsworth, 2001). Therefore, exploring the important role of PDP in improving human quality of life and treating diseases has become the focal point of pharmaceutical science and drug discovery. The isolation of plant-based products has intensified in recent years since PDP products are considered safe, easy to obtain, and economically viable with fewer side effects as compared to conventional disease treatments (Firenzuoli and Gori, 2007).

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The most common type of dementia in the elderly is Alzheimer's disease, a progressive neurodegenerative disorder. An Amaryllidaceae alkaloid, galanthamine, and a synthetic derivative of physostigmine (rivastigmine) are used for the treatment of Alzheimer's disease (Ng *et al.,* 2015; Ghane *et al.,* 2018). Many herbal compounds are also extensively studied for their anticancer properties (Patel *et al.,* 2020). Previous studies have suggested that natural compounds (namely polyphenols, flavonoids, monoterpenes, and sulphur compounds) have chemopreventive and anticancer effects by regulating apoptosis, migration, proliferation, and other mechanisms (Ma *et al.,* 2021). Many chemotherapeutic agents used in cancer treatment have been prepared from synthetic, semisynthetic, and naturally occurring compounds. There is a wide range of plant-derived compounds approved for use as anticancer agents, such as vincristine and vinblastine (indole alkaloids from *Catharanthus roseus*), taxol (diterpenoid alkaloid from *Taxus* and *Corylus* species), and camptothecin (quinoline alkaloid from *Camptotheca acuminata*) (Changxing *et al.,* 2020). There is an ongoing scientific interest in discovering and exploring effective anticancer agents from natural product sources (Ma *et al.,* 2021).

Polygonum L. is a member of the Polygonaceae family with almost 150 known species distributed worldwide (Mahmoudi *et al.,* 2020). Eight *Polygonum* species are endemic to Turkey (Keskin, 2012). In Turkey, some *Polygonum* species are consumed as vegetables, and are regionally named "madımak" (Özüdoğru *et al.,* 2011). Several *Polygonum* species are traditionally used in many countries as an astringent and for the treatment of diarrhoea, haemorrhage, fever, ulcers, lupus, some cancers; and digestive, cardiovascular, neurodegenerative, and respiratory diseases (Ayaz *et al.,* 2016; Ozturk *et al.,* 2018; Suroowan *et al.,* 2019). There are several reports available on the antioxidant, anticancer, anti-inflammatory, antimicrobial, antiviral, and neuroprotective effects of different *Polygonum* species (Dong *et al.,* 2014; Muddathir *et al.,* 2017). For example, the AChE inhibitory potential of *P. maritimum* (Rodrigues *et al.,* 2018), *P. multiflorum* (Li *et al.,* 2017), and *P. hydropiper* (Ayaz *et al.,* 2015) have been reported previously. However, to the best of our knowledge, no reports on AChE inhibitory effects of *P. aviculare, P. patulum subsp. pulchellum, P. lapathifolium,* and *P. istanbulicum* have been published. Also, apart from two studies, there is no information regarding the anticancer potential of the mentioned species on HeLa cervical cancer cell lines (Mohammad *et al.,* 2011; Lajter *et al.,* 2013).

The present work evaluated for the first time the anti-AChE activity of four *Polygonum* species, namely *P. aviculare, P. patulum subsp. pulchellum, P. lapathifolium,* and the only endemic species *P. istanbulicum* collected from Istanbul. Additionally, the antioxidant and anticancer activities as well as the total phenolic and flavonoid contents of the extracts were determined. To the best of our knowledge, this is the first study on the biological activity and phenolic content of *P. istanbulicum*. The findings of the present work are important and relevant due to the critically endangered status of *P. istanbulicum* (Keskin, 2009).

Materials and methods

Chemicals

2,2-diphenyl-1-picryl-hydrazyl (DPPH), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), α-tocopherol, acetylthiocholine iodide (ATChI), AChE, aluminium chloride ($AlCl_3$), catechin, dimethyl sulfoxide (DMSO), galantamine hydrobromide, gallic acid (GA), iron (II) sulphate heptahydrate ($FeSO_4.7H_2O$), and quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium acetate ($C_2H_3NaO_2$), sodium carbonate (Na_2CO_3), sodium hydroxide (NaOH), sodium nitrite ($NaNO_2$), ferric chloride ($FeCl_3$), and 2,4,6-tripyridyl-S-triazine (TPTZ) were purchased from Merck (Darmstadt, Germany). Fetal bovine serum (FBS), phosphate-buffered saline (PBS), and high glucose Dulbecco's modified Eagle's medium (DMEM) F-12 were purchased from Wisent Inc. (Quebec, Canada), while penicillin and streptomycin were purchased from PAA Laboratories (GmBH, Austria). All the other chemicals were of analytical grade.

Plant material

The aerial parts of the *P. aviculare* L. (41.028353 N, 28.618432 E), *P. patulum* Beib. subsp. *pulchellum* (Lois.) Leblebici (41.031181 N, 28.622669 E), *P. lapathifolium* L. (41.326790 N, 28.574907E), and *P. istanbulicum* Keskin (40.949013 N, 29.141156 E) were collected from Istanbul, Turkey between September and October 2013. The collected fresh samples were identified at the Department of Pharmaceutical Botany, Faculty of Pharmacy, Istanbul University. Each specimen was given a voucher number, and placed in the herbarium of the Faculty of Pharmacy, Istanbul University namely *P. aviculare* (ISTE 101072), *P. patulum* subsp. *pulchellum* (ISTE 101071), *P. lapathifolium*

(ISTE 101064), and *P. istanbulicum* (ISTE 101074).

Preparation of the extracts

The aerial parts of the plants were cut into small pieces, and dried on laboratory benches in the shade. A IKA-Werke/M20 grinder (Staufen, Germany) was used to ground the samples into powder. The dried powder (20 g) of the four *Polygonum* species was sequentially extracted for 8 - 10 h with chloroform and methanol using a Soxhlet apparatus with 150 mL of organic solvent. The ethanolic extracts were obtained by maceration. The dried plant was twice macerated in ethanol for three days at 25°C. The mixtures were filtered using Whatman filter paper no. 1. A rotary evaporator (Buchi/R210, Flawil, Switzerland) under pressure with a water bath at 40°C was used to evaporate the remaining solvents. The extracts were stored at -20°C until further analysis. Each *Polygonum* extract (20 mg) was dissolved in 1 mL of DMSO to reach a 20 mg/mL concentration. A stock solution (20 mg/mL) was prepared fresh daily, and stored at 4°C. Thereafter, to make a fixed amount of the dilute solution from the stock solution, a serial dilution was carried out daily. This process was repeated over the course of several days to prepare the different concentrations levels (0.0002 - 10 mg/mL) required for each test.

Determination of the total phenolic and flavonoid contents

A modified Folin-Ciocalteu method was used to estimate the total phenolic content of the extracts (Slinkard and Singleton, 1977). Briefly, 5 µL of either the extract or GA solution was added to each well of a 96-well plate containing 225 µL of distilled water. After waiting 3 min, 5 µL of 2 N Folin-Ciocalteu reagent (diluted 1:3 in distilled water) was mixed with the solution, and then, 15 µL of a 2% Na_2CO_3 solution was added. The reaction mixture was incubated at room temperature and away from light for 2 h. Subsequently, the absorbance of the mixture was measured at 760 nm against a blank. To create a calibration curve, GA solution (0.03 - 0.25 mg/mL) was used as standard. The total phenolic compounds were expressed as means mg of GA equivalents (GAE)/g extract.

To determine the total flavonoid content, a colorimetric modified aluminium chloride method was used (Kim *et al.,* 2003). Briefly, 25 µL of each extract was added to each well of a 96-well plate followed by the addition of 125 µL of distilled water. At the same time, 25 µL of a catechin solution (0.015 - 0.25 mg/mL) was added as a standard solution. Thereafter, 7.5 µL of a 5% $NaNO_2$ solution was added to the mixture. A 10% $AlCl_3$ solution (15 µL) was added after 6 min. The mixture was left to stand for 5 min, and then, 1 M NaOH solution (50 µL) followed by distilled water (27.5 µL) was added to the mixture, which was then shaken well. Finally, the absorbance of the mixture was measured at 510 nm against a blank. The results were expressed as mg of catechin equivalents (CE)/g extract.

Determination of the DPPH radical scavenging activity

To evaluate the inhibition levels of the extracts on a free radical DPPH, the method of Brand-Williams *et al.* (1995) was performed. Briefly, 10 µL of the different concentrations of the plant extracts were mixed with 240 µL of a 0.1 mM DPPH solution. Then, the mixture was allowed to settle in the dark at room temperature for 30 min. Afterward, the decrease in absorbance as a result of decolourisation from the purple DPPH radical to the yellow DPPH molecule was measured at 517 nm against methanol. Quercetin (0.01 - 0.25 mg/mL) and α-tocopherol (0.01 - 0.50 mg/mL) were used as standards, and methanol was used as a control. The percentage inhibition was calculated using Eq. 1, and the DPPH radical scavenging activity of extracts was expressed as a half-maximal effective concentration (EC_{50}).

$$\text{DPPH radical scavenging activity (\%)} = \left(1 - \frac{\text{Absorbance of sample at 517 nm}}{\text{Absorbance of control at 517 nm}}\right) \times 100 \quad \text{(Eq. 1)}$$

Determination of the ferric-reducing antioxidant power (FRAP)

The reducing power of the extracts was determined using the FRAP method with some adjustment (Benzie and Strain, 1996). The principle of this method is based on the reduction of $Fe(TPTZ)_2$(III) complex to blue $Fe(TPTZ)_2$(II) in the presence of antioxidants (Benzie and Strain, 1996). The FRAP reagent was freshly prepared before use by mixing 0.3 M acetate buffer (pH 3.6), 10 mM TPTZ solution (in 40 mM HCl), and 20 mM $FeCl_3$ solution at a ratio of 10:1:1 (v:v:v). Next, 10 µL of the different concentrations from each extract and the standard, followed by 20 µL of DMSO were added to each well. The mixture was incubated for 10 min at 37°C. Thereafter, 270 µL of the prepared FRAP reagent was added. Finally, the mixture's absorbance was measured at 593 nm immediately after incubating for 4 min at room temperature against a blank. Quercetin and α-tocopherol were

also used as standards in this method. Moreover, a $FeSO_4.7H_2O$ solution was used to create a standard curve ($y = 0.0176x + 0.0001$) at a final concentration of 8 - 50 µM. The results were presented as µM Fe^{2+} equivalents.

Determination of the AChE inhibitory activity

The AChE inhibitory activity of the extracts was determined by a modified Ellman method (Ellman *et al.,* 1961). Ellman's solution was prepared by mixing 100 mM, pH 7.5 phosphate buffer, 10 mM DTNB solution, and 75 mM ATChI solution at a ratio of 75:2.5:1 (v:v:v). Next, 20 µL of different concentrations of the extracts and 220 µL of Ellman's solution were mixed, and the reaction was initiated with the addition of 10 µL of AChE solution (0.5 U/mL). The reaction rate was monitored at 412 nm for 10 min. Galantamine was used as the standard, and distilled water was used as the control. The enzyme inhibitory activity of the extracts as a percentage was determined using Eq. 2:

$$\text{AChE inhibitory activity (\%)} = \left(1 - \frac{\text{Reaction rate of sample at 412 nm}}{\text{Reaction rate of control at 412 nm}}\right) \times 100 \quad \text{(Eq. 2)}$$

Cell culture

The cytotoxicity of the extracts was assessed using normal rat kidney proximal epithelial cells (NRK-52E; CRL-1571) from the American Type Cell Culture (ATCC) and HeLa (Henrietta Lacks; CCL-2) cervical cancer cells from the Department of Genetics, Istanbul University. The cells were cultured in DMEM F-12 supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. Cells were checked under an inverted microscope (CKX41, Olympus Co., USA), and subculturing was performed every three to four days until the cells reached 70 - 80% confluence (Abudayyak *et al.,* 2015).

Determination of the anticancer activity

The anticancer activity of the extracts was evaluated using the MTT method with some modifications (Mosmann, 1983; Alley *et al.,* 1988). The cells were counted using a trypan blue solution. Next, 100 µL of cell suspension (10^4 cells per well) was plated into 96-well plates. The plates were incubated to allow for cell attachment at 37°C. After 24 h, the cells were treated with serial concentrations (0.25 - 25 µg/mL) of the extracts (10 µL), and the plates were incubated for 24 h at 37°C. Then, the exposed cells were washed with PBS buffer, and added to 200 µL of fresh culture medium in individual wells. Then, 20 µL of MTT solution (5 mg/mL in PBS) was added to each well, and incubated at 37°C for 1 h. Following incubation, the supernatant containing MTT was discarded, and the resulting formazan crystals were solubilised in 100 µL of DMSO, and shaken for 5 min at 150 rpm. After the addition of DMSO, the absorbance was read at 590 nm using a microplate reader (BioTek Epoch, Winooski-USA). Stock solutions of the extracts were prepared by dissolving in DMSO, and the serial dilutions were freshly made in the cell culture medium. The medium containing 1% DMSO served as the control. The cytotoxic activity was measured according to Eq. 3. The results were expressed as IC_{50} values (the inhibitory concentration required to reduce cell growth by 50%).

$$\text{Cytotoxic activity (\%)} = \left(1 - \frac{\text{Absorbance of sample at 590 nm}}{\text{Absorbance of control at 590 nm}}\right) \times 100 \quad \text{(Eq. 3)}$$

Statistical analysis

All the results were expressed as the means of three replicates ± standard deviation (SD). A student's *t*-test was used for comparison between two means using the NCSS statistical computer package. Mean values were considered significantly different when $p < 0.05$.

Results and discussion

Total phenolic and flavonoid content

Phenolic compounds are secondary metabolites that are good antioxidants due to their metal-chelating potential and redox properties that include reducing agents, hydrogen donors, and singlet oxygen quenchers. Flavonoids are antioxidant compounds that act as free radical scavengers (Patel *et al.,* 2018). In the present work, the total phenolic content of the *Polygonum* extracts were detected and expressed as mg GAE/g extract ($y = 0.9175x - 0.003$). As shown in Table 1, the ethanolic extracts have the highest phenolic content (ranged from 88.3 ± 5.53 to 207.03 ± 14.12 mg GAE/g extract) followed by the methanolic extracts (ranged from 76.81 ± 4.12 to 138.63 ± 10.28 mg GAE/g extract). However, the chloroform extracts exhibited the lowest (ranged from 22.33 ± 3.05 to 58.61 ± 2.01 mg GAE/g extract). *P. istanbulicum* and *P. patulum* subsp. *pulchellum* possessed the highest phenolic contents for all solvent, followed by *P. lapathifolium* and *P. aviculare*. Similar to our results, Cai *et al.* (2020) showed that *P. aviculare* leaves extracted with 70%

Table 1. Total phenolic and flavonoid contents of *Polygonum* extracts.

	Total phenolic content (mg GAE/g extract)			Total flavonoid content (mg CE/g extract)		
	Ethanol	**Methanol**	**Chloroform**	**Ethanol**	**Methanol**	**Chloroform**
P. aviculare	88.31 ± 5.53[a]	76.81 ± 4.12[a]	33.88 ± 1.56[a]	56.23 ± 4.42[a]	60.02 ± 5.11[a]	21.92 ± 2.69[a]
P. patulum subsp. *pulchellum*	122.94 ± 8.86[b]	138.63 ± 8.28[b]	58.61 ± 2.01[b]	80.61 ± 8.07[b]	104.26 ± 13.51[b]	38.42 ± 2.36[b]
P. lapathifolium	90.39 ± 7.55[a]	98.94 ± 10.46[c]	22.33 ± 3.05[c]	70.26 ± 0.88[b]	72.35 ± 6.02[a]	11.66 ± 0.36[c]
P. istanbulicum	207.03 ± 14.12[c]	138.15 ± 10.28[b]	40.71 ± 4.66[a]	124.95 ± 7.84[c]	102.09 ± 5.37[b]	37.33 ± 2.26[b]

Values are mean ± SD of three replicates ($n = 3$). Different lowercase superscripts in the same column indicate significant difference ($p < 0.05$).

ethanol had higher phenolic content (18.50 ± 0.66 mg GAE/g DW) than the other extracts (water and ethyl acetate). It has also been reported that methanolic leaf extracts of *P. minus* had the highest phenolic content (174.00 ± 0.18 mg GAE/g extract) among the other extracts (hexane, ethyl acetate, and water) (Abdullah *et al.,* 2017). This value was close to our result for the methanolic extracts of *P. istanbulicum*. However, in another study (Azlim Almey *et al.,* 2010), the methanolic extract of *P. minus* leaves provided a lower phenolic content (31.38 ± 0.13 GAE mg/g) than reported by Abdullah e*t al.* (2017).

Total flavonoid content of the extract was expressed as mg CE/g extract ($y = 2.3096x + 0.0066$). All the ethanolic and methanolic extracts contained a significant amount of flavonoid which varied from 56.23 ± 4.42 to 124.95 ± 7.84 mg CE/g extract (Table 1). Ethanolic extract of *P. istanbulicum* exhibited the highest ($p < 0.05$) total flavonoid content followed by the methanolic extract of *P. patulum* subsp. *pulchellum*. The results revealed that chloroform was the least effective solvent for extracting flavonoids. It is apparent from the results that the ethanolic extract of *P. istanbulicum* includes the highest amount of phenolic and flavonoid contents. This is in agreement with the yield of the extraction where the ethanolic extract of *P. istanbulicum* (14.5%) produced the highest yield in comparison with methanolic (11.6%) and chloroform (10.6%) extracts (Table 2). The total flavonoid content of the other *Polygonum* species have previously been reported, for example the methanolic leaf extract of *P. minus* (53.19 ± 0.71 mg QE/g extract) (Abdullah *et al.,* 2017), the ethanolic (70%) leaf extract of *P. aviculare* (19.78 ± 0.67 mg RE/g DW) (Cai *et al.,* 2020), and the ethanolic (50%) extract of *P. aviculare* (112.7 ± 13 mg QE/g sample) (Hsu, 2006). With regard to the ethanolic extract of *P. aviculare*, the total phenolic and flavonoid contents obtained in the present work were lower as compared to the values reported by Hsu (2006), although they were higher than the values reported by Cai *et al.* (2020). This variation could be due to the application of different extraction methods and assay procedures or variations in the plants' origin. In the study of Hsu (2006), dried *P. aviculare* was purchased from a local drugstore in Taiwan; while in the study of Cai *et al.* (2020), *P. aviculare* was collected from Shandong, China. Nevertheless, the results of the present work suggest that the tested *Polygonum* species, especially *P. istanbulicum*, could be a good source of phenolics and flavonoids. The chemical profiles of many *Polygonum* species have been investigated, and it has been reported that these species are rich in phenolic and flavonoid components.

Table 2. Extraction yield (%) of *Polygonum* extracts.

	Extraction yield (%)		
	Ethanol	**Methanol**	**Chloroform**
P. aviculare	18.2	10.4	9.13
P. patulum subsp. *pulchellum*	29.1	21.8	6.5
P. lapathifolium	14.3	16.9	8.9
P. istanbulicum	14.5	11.6	10.6

Smolarz (2002) reported that *P. hydropiper, P. bistorta, P. aviculare, P. persicaria, P. lapathifolium, P. amphibium*, and *P. convolvulus* contain flavonoid aglycones, such as taxifolin, quercetin, and kaempferol. Mahmoudi *et al.* (2019) reported that (+)-catechin was the major flavonoid compound in the methanolic *P. equisetiforme* extract followed by quercetin-3-*O*-galactoside, epicatechin, quercetin-3-*O*-rhamnoside, and cirsiliol. Cai *et al.* (2020) showed that gallic acid, hyperoside, avicularin, 3″-*O*-galloylmyricitrin, and quercitrin are the most abundant phenolic components in *P. aviculare*.

DPPH radical scavenging activity

The ability of the methanolic, ethanolic, and chloroform extracts of four *Polygonum* species to scavenge DPPH radicals was determined. The results confirmed dose-dependent DPPH radical scavenging potential for all the extracts. The antioxidant activity expressed as EC_{50} values is shown in Table 3. The ethanolic and methanolic extracts of the plants exhibited higher antioxidant activity than the chloroform extract. Among the four plants and the three different extracts, the ethanolic extract of *P. istanbulicum* possessed the highest ($p < 0.05$) capacity to inhibit DPPH radicals (EC_{50}, 8.09 ± 0.50 µg/mL), while the lowest was exhibited by the chloroform extract of *P. lapathifolium* (EC_{50}, 218.44 ± 24.46 µg/mL). In this assay, final extract concentrations in the reaction mixture were 1.5 - 25 µg/mL for *P. istanbulicum* and 25 - 400 µg/mL for *P. lapathifolium*. The DPPH radical scavenging activity of the ethanolic extract from *P. istanbulicum* was comparable to the positive control, α-tocopherol (EC_{50}, 8.90 ± 0.44 µg/mL), but it was not as effective as quercetin (EC_{50}, 2.66 ± 0.03 µg/mL).

In the present work, the free radical scavenging activity of *P. istanbulicum* is reported for the first time. The obtained results of *P. aviculare* and *P. patulum* subsp. *pulchellum* from the DPPH scavenging assay are consistent with previous studies (Hsu, 2006; Shahraki, 2013). Luo *et al.* (2018) reported that the ethyl acetate extract of *P. aviculare* was rich in quercetin. In another study, it was reported that the ethanolic extract of *P. bistorta* contains numerous phenolic acid and flavonoid compounds such as quercetin, kaempferol, luteolin derivatives, and caffeic and chlorogenic acids (Pirvu *et al.,* 2017). In the present work, a high correlation was observed between the total phenolic content and the DPPH radical scavenging activities of *P. aviculare* (r = -0,983), *P. patulum* subsp. *pulchellum* (r = -0,989), *P. lapathifolium* (r = -0,996), and *P. istanbulicum* (r = -0,949) extracts. Considering the correlation and the previous phenolic profile studies, it can be suggested that the phenolic compounds in the *Polygonum* extracts may play a prominent role in scavenging radicals. Similar to our findings, Abdullah *et al.* (2017) reported that the total phenolic content of the *P. minus* leaf extracts showed a strong correlation ($r > 0.8$) with ABTS and the DPPH free radical scavenging activities.

Ferric-reducing antioxidant power (FRAP)

The FRAP values of the extracts were presented as µM of Fe^{2+} equivalent at 20 µg/mL concentration (Table 4). The ethanolic extract of *P. istanbulicum* with a FRAP value of 88.19 ± 3.89 µM Fe^{2+} demonstrated the highest ($p < 0.05$) reducing activity, comparable to the reducing activity of α-tocopherol (89.33 ± 2.56 µM Fe^{2+}) at the same concentration (20 µg/mL). The highest FRAP value in the methanolic extracts was expressed by

Table 3. DPPH radical scavenging activities of *Polygonum* extracts and standards.

	EC_{50} (µg/mL)*		
	Ethanol	**Methanol**	**Chloroform**
P. aviculare	25.72 ± 0.98^{a}	27.95 ± 5.05^{a}	137.30 ± 2.92^{a}
P. patulum subsp. *pulchellum*	16.22 ± 1.24^{b}	13.86 ± 2.07^{b}	65.17 ± 7.03^{b}
P. lapathifolium	25.87 ± 1.29^{a}	23.43 ± 4.98^{a}	218.44 ± 24.46^{c}
P. istanbulicum	8.09 ± 0.50^{c}	15.11 ± 0.84^{b}	69.18 ± 10.16^{b}
Quercetin	2.66 ± 0.03^{d}		
α-tocopherol	8.90 ± 0.44^{c}		

Values are mean ± SD of three replicates (n = 3). Different lowercase superscripts in the same column indicate significant difference ($p < 0.05$). EC_{50} is the effective concentration of the extract or standard required to scavenge DPPH radicals by 50%. EC_{50} values were obtained by interpolation from the linear regression analysis. *All concentrations are the final extract/standard concentrations in the reaction mixture.

P. patulum subsp. *pulchellum* (70.49 ± 11.11 µM Fe^{2+}) and *P. istanbulicum* (66.04 ± 7.38 µM Fe^{2+}). Based on the results from Table 4, the chloroform extracts possessed the least reducing activity. Similar to our result, Shahraki (2013) reported that the chloroform fraction of *P. patulum* had the lowest FRAP value (1.75 ± 0.1 mol Fe^{2+}/g) as compared to the crude methanolic extract (2.11 ± 0.15 mol Fe^{2+}/g), water (1.96 ± 0.05 mol Fe^{2+}/g), and ethyl acetate (12.8 ± 0.13 mol Fe^{2+}/g) portions. Also, Chan *et al.* (2010) reported that extracts prepared from polar solvents had higher FRAP values than extracts prepared from less polar solvents. Similarly, chloroform was the least polar solvent used in the present work.

It has been reported that polyphenols have a higher antioxidant activity than ascorbic acid and α-tocopherol according to the FRAP assay (Pulido *et al.,* 2000). Maizura *et al.* (2011) reported that the high reducing ability of *P. minus* correlated with the phenolic content. Similarly, in another study with *P. glabrum*, the high FRAP value of the plant was explained by the fact that it had a high total phenolic content (Muddathir *et al.,* 2017). It can be deduced that the potentially high reducing activity of the *Polygonum* extracts (primarily the ethanolic extract of *P. istanbulicum*) was mainly due to the presence of phenolic compounds in the extracts. In accordance with our findings, Chan *et al.* (2010) reported that the total phenolic content of the *P. multiflorum* extracts showed a significant positive correlation with the FRAP values ($r > 0.7$).

AChE inhibitory activity

AChE is an enzyme that hydrolyses neurotransmitter acetylcholine, and the use of AChE inhibitors is an effective strategy for treating neurological disorders like Alzheimer's disease (Attar and Ghane, 2019). The AChE inhibitory activities of the tested *Polygonum* species are presented in Table 5. All extracts exhibited AChE inhibitory activity in a concentration-dependent manner. Both the ethanolic and methanolic extracts of *P. istanbulicum* and *P. patulum* subsp. *pulchellum* displayed the strongest inhibitory activity against AChE at 400 µg/mL (> 80%), followed by the ethanolic extracts of *P. aviculare* (75.59 ± 2.24%) and *P. lapathifolium* (60.55 ± 2.76%). However, the chloroform extracts of the tested plants showed moderate inhibitory activity against AChE (ranged from 32.19 ± 2.09 to 48.34 ± 3.41%, at 400 µg/mL). The high AChE inhibitory potency of the ethanolic and methanolic extracts was comparable to the reference drug galantamine. The inhibitory profile of the extracts may result from the high concentration of flavonoids. Many phenolic phytochemicals have been proposed as potential therapeutics for their cognitive effects (Howes and Perry, 2011). Our results are comparable to previous studies that showed a strong AChE inhibitory profile of the leaf extracts from *P. maritimum* (IC_{50}, 0.27 ± 0.01 mg/mL for methanolic extract, and 0.91 ± 0.02 for dichloromethane extract) (Rodrigues *et al.,* 2018) and the ethanolic root extract from *P. multiflorum* (83.07 ± 0.68% at 200 µg/mL) (Li *et al.,* 2017). Similarly, Ayaz *et al.* (2015) showed that the essential oils from the leaves of *P. hydropiper* displayed concentration-dependent inhibition on AChE (63.66 ± 0.33% at 250 µg/mL). These results indicate that the *Polygonum* species may provide a new source of natural anti-Alzheimer compounds. Furthermore, it has been reported that different herbal drug products from the *Polygonum* species have significant effects on improving dementia

Table 4. Reducing power of *Polygonum* extracts and standards.

	FRAP values (µM Fe^{2+})*		
	Ethanol	**Methanol**	**Chloroform**
P. aviculare	33.59 ± 2.34[a]	32.04 ± 3.58[a]	9.06 ± 0.63[a]
P. patulum subsp. *pulchellum*	51.93 ± 1.36[b]	70.49 ± 11.11[b]	19.84 ± 1.76[b]
P. lapathifolium	32.02 ± 1.75[a]	39.10 ± 5.03[a]	7.59 ± 1.05[a]
P. istanbulicum	88.19 ± 3.89[c]	66.04 ± 7.38[b]	13.48 ± 1.19[c]
Quercetin	94.74 ± 3.76[c]		
α-tocopherol	89.33 ± 2.56[c]		

Values are mean ± SD of three replicates ($n = 3$). Different lowercase superscripts in the same column indicate significant difference ($p < 0.05$). *FRAP values of the *Polygonum* extracts and α-tocopherol at 20 µg/mL, and quercetin at 4 µg/mL of final concentrations in the reaction mixture.

Table 5. Acetylcholinesterase inhibitory activities (% inhibition and EC_{50} values) of *Polygonum* extracts and standards.

	Concentration (µg/mL)*	% inhibition		
		Ethanol	Methanol	Chloroform
P. aviculare	400	75.59 ± 2.24	41.24 ± 4.01	38.85 ± 0.79
	200	57.69 ± 2.70	29.27 ± 2.27	33.16 ± 1.52
	100	45.97 ± 1.59	19.6 ± 0.92	ND
	50	38.87 ± 1.94	ND	ND
	EC_{50} (µg/mL)	143.32 ± 6.63[a]	ND	ND
P. patulum subsp. *pulchellum*	400	82.96 ± 1.32	81.29 ± 3.77	46.71 ± 1.33
	200	66.99 ± 2.73	62.69 ± 2.95	32.73 ± 1.47
	100	54.30 ± 1.85	38.61 ± 3.45	ND
	50	42.36 ± 3.24	18.03 ± 0.85	ND
	EC_{50} (µg/mL)	80.97 ± 6.08[b]	186.79 ± 13.93[a]	ND
P. lapathifolium	400	60.55 ± 2.76	35.86 ± 2.06	32.19 ± 2.09
	200	40.07 ± 2.10	18.03 ± 1.36	18.29 ± 2.55
	100	33.07 ± 1.64	ND	ND
	100	ND	ND	ND
	EC_{50} (µg/mL)	293.19 ± 18.96[c]	ND	ND
P. istanbulicum	400	88.23 ± 3.44	83.43 ± 1.09	48.34 ± 3.41
	200	71.54 ± 2.76	55.22 ± 0.84	34.56 ± 1.07
	100	63.25 ± 3.68	44.49 ± 3.93	ND
	50	44.73 ± 2.59	19.06 ± 1.50	ND
	EC_{50} (µg/mL)	56.76 ± 6.64[d]	184.11 ± 4.99[a]	ND
Galantamine[#]	4	88.17 ± 0.62		
	2	78.58 ± 1.80		
	1	65.32 ± 2.62		
	0.5	47.69 ± 2.96		
	EC_{50} (µg/mL)	0.70 ± 0.04[e]		

Values are mean ± SD of three replicates (n = 3). Different lowercase superscripts in the same column indicate significant difference ($p < 0.05$). EC_{50} is the effective concentration of extract or standard required to inhibit enzyme activity by 50%. *All concentrations are the final extract concentrations in the reaction mixtures. [#]Galantamine dissolved in distilled water (0.25 - 4 µg/mL of final concentrations in the reaction mixture). ND: not detected.

(May *et al.,* 2009).

Cytotoxic activity

The results of the cytotoxicity screening of the *Polygonum* extracts against NRK-52E and HeLa cell lines are summarised in Table 6. Among the tested plants, the cytotoxic effect of *P. patulum* subsp. *pulchellum* and *P. istanbulicum* against NRK-52E and HeLa cancer cell lines was investigated for the first time.

Table 6. Cytotoxic activities of *Polygonum* extracts against NRK-52E and HeLa cell lines.

	IC_{50} (µg/mL)					
	Ethanol		**Methanol**		**Chloroform**	
	HeLa	**NRK-52E**	**HeLa**	**NRK-52E**	**HeLa**	**NRK-52E**
P. aviculare	12.22 ± 1.46^{a}	NT	11.53 ± 2.67^{a}	9.87 ± 1.38^{a}	NT	11.81 ± 2.61^{a}
P. patulum subsp. *pulchellum*	9.70 ± 0.75^{ab}	NT	11.47 ± 2.49^{a}	7.49 ± 1.56^{a}	NT	13.70 ± 3.82^{a}
P. lapathifolium	8.70 ± 1.35^{b}	NT	8.65 ± 2.92^{a}	4.81 ± 0.98^{b}	NT	NT
P. istanbulicum	10.26 ± 2.36^{ab}	NT	23.09 ± 3.82^{b}	15.05 ± 2.75^{c}	NT	5.61 ± 1.26^{b}

Values are mean ± SD of three replicates ($n = 3$). Different lowercase superscripts in the same column indicate significant difference ($p < 0.05$). IC_{50} is the concentration of the extract required to inhibit cell growth by 50%. NT: non-toxic; NRK-52E: normal rat kidney epithelium; and HeLa: human cervical cancer.

All the ethanolic extracts showed selective cytotoxic activity against HeLa cancer cell line, and the plant with the most cytotoxic activity against the cell was *P. lapathifolium* (IC_{50}, 8.70 ± 1.35 µg/mL). As can be seen in Table 6, the ethanolic extract of *P. aviculare* showed selective cytotoxicity against HeLa cancer cells, and the results support the findings related to the cytotoxic activity of *P. aviculare* against different cell lines (Habibi *et al.,* 2011; Mohammad *et al.,* 2011). Ethnobotanical studies reported that the leaves of *P. aviculare* are used for cancer treatment (Suroowan *et al.,* 2019). Our results seem to be compatible with the traditional use of the plant.

The methanolic extract of the plants showed non-selective cytotoxic activity. When the methanolic extracts were compared, it was seen that the methanolic extract of *P. lapathifolium* was the most cytotoxic extract toward the cancer cells (IC_{50}, 8.65 ± 2.92 µg/mL). However, this extract also showed cytotoxic activity toward the healthy cell line (IC_{50}, 4.81 ± 0.98 µg/mL). Lajter *et al.* (2013) observed that the herbal chloroform extract of *P. lapathifolium* was not cytotoxic against HeLa cell lines, whereas the root chloroform extract of *P. lapathifolium* showed moderate cytotoxicity against HeLa cells with 47.66 ± 1.14% cell death at 30 µg/mL.

The methanolic extract of *P. istanbulicum* displayed minimal cytotoxicity against both normal and cancer cells. Also, it was seen that all chloroform extracts did not show cytotoxicity against HeLa cells, whereas they did exhibit cytotoxic activity against normal cells.

In the literature, there are studies that show the cytotoxic effect of other *Polygonum* species. Ayaz *et al.* (2019) reported that *P. hydropiper* displayed cytotoxicity against HeLa, MCF-7, and NIH/3T3 cells, and this effect could be due to the steroidal compounds such as β-sitosterol and stigmasterol in the plant.

Conclusion

This work presented the total phenolic and flavonoid contents, and the antioxidant, anti-AChE, and anticancer activities of *P. aviculare, P. patulum* subsp. *pulchellum, P. lapathifolium*, and the only endemic species *P. istanbulicum*. Biological activity of the endemic *P. istanbulicum* was evaluated for the first time in the present work. Among the four *Polygonum* species, *P. istanbulicum* exhibited the highest amounts of total phenolics and flavonoids as well as the best antioxidant and anti-AChE activity. The ethanolic extracts of the tested plants showed selective cytotoxic activity against HeLa cervical cancer cell lines. Ethanol was the most efficient solvent for extracting the phenolics as well as the biologically active compounds. Results suggest that the ethanolic extract of *P. istanbulicum* may be a potential source of substances with antioxidant and anti-AChE properties. Further studies are nevertheless required to identify the bioactive components present in the plants, and to fully understand the mechanisms of their activity.

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