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Original Article

Evaluation of Chemical Composition and *In-vitro* Biological Activities of Three Endemic *Hypericum* Species from Anatolia (*H. thymbrifolium, H. spectabile* and *H. pseudolaeve*)

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Abstract

In the present work we carried out a phytochemical and biological investigation on three endemic *Hypericum* species, *i.e. Hypericum thymbrifolium (H. thymbrifolium), Hypericum spectabile (H. spectabile) and Hypericum pseudolaeve (H. pseudolaeve)* from Anatolia in order to discover new sources of natural compounds for the treatment of inflammatory and neurodegenerative disorders. HPLC-DAD analysis indicated that two naphthodianthrones (pseudohypericin and hypericin) together with chlorogenic acid, rutin, hyperoside, isoquercitrin, kaempferol, quercitrin, quercetin, amentoflavone, and hyperforin are the main compounds present in the methanol extracts. After chemical characterization, all extracts were in-vitro biologically assayed for antioxidant potential by lipid peroxidation inhibitory activity, DPPH, FRAP assays, and superoxide radical scavenging activity, for AChE inhibitory activity by Ellman's method, for COX inhibitory activity by using enzyme immunoassay (EIA) kit, for cytotoxic activity on HeLa and NRK-52E cell lines by MTT assay. The superoxide radical scavenging activity and lipid peroxidation inhibitory activity of *H. spectabile* (EC_{50} = 0.430 mg/mL) were more remarkable than that of *H. thymbrifolium* and *H. pseudolaeve.* The extracts showed moderate inhibitory activity on AChE (from 49.37% to 63.41%). The best inhibitory activity against COX-1 (71.77% and 77.04%, respectively) and COX-2 (64.14% and 72.23%, respectively) were shown by *H. thymbrifolium* and *H. spectabile,* which may be due to their richest chlorogenic acid content (0.29576% and 0.23567%, respectively). Cytotoxicity screening results showed that the extracts did not demonstrate significant cytotoxic activity. It was concluded that the most promising extract with antioxidant, anti-inflammatory, and AChE inhibition potential is *H. spectabile.*

Keywords: *Hypericum*; Endemic; HPLC; Chemical composition; Cyclooxygenase inhibition; Alzheimer's disease.

Introduction

The genus *Hypericum* L. (*Hypericaceae*) has been used for centuries for the treatment of burns, bruises, swelling, inflammation, and anxiety, as well as bacterial and viral infections. Locally, it is traditionally used both externally (as a cream or oil extract) and internally (as a tea) with many therapeutic applications (1-4). *Hypericum* has

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nearly 465 species all over the world and is represented by nearly 100 taxa grouped under 19 sections in Turkey, among them, 45 taxa are endemic (5-7).

For the last few years, there has been an increasing biological activity trend and awareness in *Hypericum* research. Quite a significant amount of research has already been carried out in exploring the chemistry of different parts of *Hypericum* (8-15). The pharmacological studies showed that this species have several activities, namely, antidepressant, anti-inflammatory, antimicrobial, antiviral, antinociceptive, and wound healing (5). In the recent years, anti-depressant applications of *Hypericum* medical products have become increasingly popular (16). The antidepressant activity was first attributed to hypericins (naphthodianthrone derivatives), but recent pharmacological and clinical results focus on hyperforins (phloroglucinol derivatives) as the main active ingredients of the extract. *Hypericum perforatum* L. (St. John's wort) preparative forms have recently gained popularity as an alternative treatment for mild to moderate depression (17). It is heartening to see that a traditional plant medicine has now led to several therapeutically useful preparations, which encourage the scientists to explore more information about this medicinal plant. It has been reported that *Hypericum* species contain variety of phenolic compounds and represent good sources of antioxidants which increase their usability potential in ethnomedicine (9, 13). Numerous antioxidant investigations have been carried out on *Hypericum* species (18-26).

It has been reported that *Hypericum* species have anti-inflammatory activity *in-vitro* and in different animal models of edema possibly due to inhibition of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression (27, 28). COX-2 is responsible for the production of pro-inflammatory mediators, prostaglandins, at the inflammatory site (29). Recognition of COX-2's key role in inflammation led to the hypothesis that it may represent a primary target for non-steroidal anti-inflammatory drugs (NSAIDs) in Alzheimer's disease (AD), consistent with inflammatory processes occurring in AD brain (30).

Cholinesterase inhibitors are the first-line treatment for AD. Acetylcholinesterase (AChE) inhibitory potential of some *Hypericum* species has been reported in previous studies (31-35).

Analysis of the cytotoxicity and anticancer cell proliferation activity was conducted in a variety of *Hypericum* species (36). It was reported that *H. perforatum* has no cytotoxic potential and oral consumption by humans is safe (37).

Considering the important role of oxidative stress and inflammation in the pathogenesis of neurological diseases such as AD, and the growing evidence of the presence of compounds with antioxidant, anti-inflammatory and AChE inhibitory potential in different *Hypericum* species; the aim of the present study was to investigate the chemical profiles and antioxidant, anti-inflammatory, anti-AChE and cytotoxic potential of the extracts from three endemic *Hypericum* species (*Hypericum thymbrifolium* Boiss. and Noë, *Hypericum spectabile* Jaub. and Spach., *Hypericum pseudolaeve* Robson) of the Turkish flora. Our research is the first report to study the phytochemical profiles and biological activities in these species.

Experimental

Chemical agents

Hypericin, chlorogenic acid, rutin, hyperoside, isoquercitrin, quercitrin, kaempferol, quercetin, amentoflavon, hyperforin, $AlCl_3$, and D-galactose were obtained from Sigma-Aldrich (Taufkirchen, Germany). Pseudohypericin was obtained from PhytoPlan (Heidelberg, Germany). Milli-Q ultrapure water was obtained from Millipore (Billerica, MA, USA), HPLC grade acetonitrile, methanol, ethyl acetate and sodium dihydrogen phosphate dihydrate were obtained from Merck (Darmstadt, Germany) and ortho-phosphoric acid 85% was obtained from Fluka (Buchs, Switzerland).

Nitroblue tetrazolium (NBT), β-nicotinamide adenine dinucleotide reduced (β-NADH), soybean L-α-phosphatidylcholine type IV-S, quercetin and catechin were purchased from Fluka (Buchs, Switzerland). Phenazine methosulphate (PMS), 2,2-diphenyl-1-picryl-hydrazyl (DPPH), gallic acid, ascorbic acid, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), acetylthiocholine

iodide (ATChI), AChE, galantamine hydrobromide were obtained from Sigma-Aldrich (St. Louis, MO, USA). 2,4,6-tripyridyl-s-triazine (TPTZ), trichloroacetic acid (TCA), thiobarbituric acid (TBA) and ferric chloride were obtained from Merck (Darmstadt, Germany). Enzyme immunoassay (EIA) kit and aspirin were obtained from Cayman Chemical (Ann Arbor, MI, USA).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was purchased from Sigma (St. Louis, MO, USA). Dimethylsulfoxide (DMSO), trypsin was purchased from (Biomatik, Canada). Ethylenediamine tetra acetic acid (EDTA), sodium hydroxide was purchased from Merck (Darmstadt, Germany). Fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM), penicillin-streptomycin, phosphate buffer saline (PBS) were purchased from Multicell-Wisent Inc. (Quebec, Canada). Cytotoxicity detection kit (LDH) which containing catalyst, dye solution and stop solution were purchased from Roche (Mannheim, Germany). All other reagents were of analytical grade.

Plant material

During the field investigations conducted in June 2010, specimens of flowering aerial parts of *H. thymbrifolium, H. spectabile* and *H. pseudolaeve* were gathered from their natural habitats on the roadsides nearby the town of Malatya located in the East Anatolia Reagion of Turkey: *H. thymbrifolium* (Malatya: Malatya to Darende, 10 km to Gürün, 1425 m), *H. spectabile*: (Malatya: Arapgir to Kemaliye, 40 km to Kemaliye, 1157 m) and *H. pseudolaeve* (Malatya: Malatya to Arapgir, 20 km to Arapgir, 1264 m). The plant materials were identified by Prof. Dr. Şükran Kültür and voucher specimens were deposited in the Herbarium of the Istanbul University Faculty of Pharmacy, Istanbul, Turkey (ISTE 93194, 93192 and 93193, respectively).

Preparation of the extracts

The samples were air-dried at room temperature under shade. The dried flowering aerial parts (10 g) of the species were macerated in methanol (100 mL) for 3 days at room temperature at dark and the resulting extract was filtered through Whatman No-1. The residue from the filtration was extracted again twice using the same procedure. The filtrates were combined and then evaporated to dryness under reduced pressure at a temperature below 45 °C. The crude methanol extract was lyophilized and stored at -20 °C (38, 39). The extracts prepared with this procedure were used in the HPLC analysis and biological activity studies.

HPLC analysis

Preparation of the standards

The calibration curves were prepared with analytical standards at the different concentration in methanol. The experiment was conducted three times providing the same conditions. The calibration curves were constructed by using average of peak areas and at least five different standard concentrations.

Preparation of the samples

The crude methanol extract was dissolved in mixture of methanol/water (8:2, v/v) (40). All samples were filtered through a 0.45 µm filter into a vial for HPLC analysis. Each sample was prepared and injected three times.

Chromatographic HPLC conditions

The *Hypericum* species have been analyzed by reversed phase HPLC coupled with DAD (HPLC-DAD). The HPLC system consisted of a Shimadzu 10A model (DAD: SPD-M10A), pump: LC-10AD and an autosampler: SIL-10AD.

The separation was accomplished on an ACE C18 (250 × 4.6 mm, particle size 5 µm) (Advanced Chromatography Technologies, Alberdeen, Scotland) column. The elution conditions were as follows: flow rate: 1 mL/min; column temperature: 40 °C; injection volume: 10 µL; detection: 590 nm for pseudohypericin and hypericin, 360 nm for phenolic compounds and 275 nm for hyperforin.

The solvent system was used as an isocratic to identify and quantitate pseudohypericin and hypericin. Separation was carried out using solvent A [ethyl acetate/15.6 g/L sodium dihydrogen phosphate adjusted to pH 2 with phosphoric acid/methanol (39:41:160, v/v/v)]. The solvent system was used as a gradient to

identify and quantitate phenolic compounds and hyperforin. The mobile phase consisted of solvent A (0.3% formic acid in water (v/v) and solvent B (0.3% formic acid in acetonitrile (v/v). The following gradient was applied: 0-8 min, 82% A; 8-18 min, 82-47% A; 18-18.1 min, 47-3% A; 18.1-29 min, 3% A; 29-40 min, 3-82% A (European Pharmacopoeia, 2008). All solvents were filtered through a 0.45 µm filter prior to use and degassed in an ultrasonic bath.

The control of the system and the data analysis procedure were performed with Shimadzu LC Solutions software.

Determination of total phenolic compounds

Total soluble phenolics in the methanolic extracts were determined with Folin-Ciocalteu reagent according to the method of Slinkard and Singleton with some modifications (41). The amount of total phenolic compounds was calculated from the calibration curve of gallic acid standard solution (covering the concentration range between 0.05 and 0.4 mg/mL) and expressed as mg gallic acid equivalents (GAE)/g dry weight (DW) of the plant material.

Determination of total flavonoid content

Total flavonoid content was determined by using a method described by Sakanaka *et al.* (2005). Total flavonoid contents were calculated from the calibration curve prepared with catechin standard solution and expressed mg of (+)-catechin equivalents (CE) per g of DW of the plant material.

Determination of antioxidant activity

Quercetin was used as reference antioxidant for the antioxidant activity assays.

Inhibition of lipid peroxidation (LPO)

LPO assay was based on the method described by Duh *et al.* (42). The formation of LPO products was assayed by the measurement of malondialdehyde (MDA) levels on the basis of MDA reacted with TBA at 532 nm according to Buege and Aust (43). The percentage inhibition of LPO was calculated by comparing the results of the sample with those of controls not treated with the extract using the following Equation:

Inhibition effect (%) = (1 − absorbance of sample at 532 nm/absorbance of control at 532 nm) × 100.

DPPH radical scavenging activity

The DPPH radical scavenging activity of the methanolic extracts was measured according to the procedure described by Brand-Williams *et al*. (44). The ability to scavenge DPPH radical was calculated by the following Equation:

DPPH radical scavenging activity (%) = (1 − absorbance of sample at 517 nm/absorbance of control at 517 nm) × 100.

Superoxide radical scavenging activity

The effects of the methanolic extracts on generation of superoxide radicals were determined by the NBT reduction method (45). The abilities to scavenge the superoxide radical were calculated by comparing the results of the sample with those of controls not treated with the extract using the following Equation:

Superoxide radical scavenging activity (%) = (1 − absorbance of sample at 560 nm/absorbance of control at 560 nm) × 100.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was carried out according to the procedure of Benzie and Strain (46). The standard curve was constructed using iron sulphate heptahydrate solution (125-2000 µM), and the results were expressed as mM Fe^{2+} equivalents.

Determination of AChE inhibitory activity

The extracts were screened for their AChE inhibitory activity through the modified Ellman›s spectrophotometric method (47). Galantamine hydrobromide was used as a standard and tested in a concentration range between 12.5 to 100 µg/mL (33.75 to 270 µM). Any increase in absorbance due to the spontaneous hydrolysis of substrate was corrected by subtracting the rate of the reaction before adding the enzyme from the rate after adding the enzyme.

AChE Inhibition (%) = (1 – reaction rate

of sample at 412 nm/reaction rate of negative control at 412 nm) × 100.

Determination of COX inhibitory activity

The ability of the extracts to inhibit ovine COX-1 and COX-2 was determined by calculating percent inhibition of prostaglandin production using EIA kit (Catalogue No. 560101, Cayman Chemical) according to the manufacturer›s instructions. Aspirin was used as a standard.

In-vitro cytotoxic activity

Cytotoxicity of *Hypericum* species at various concentrations was determined on human cervix adenocarcinoma (HeLa, ATCC® CCL-2™) and normal rat kidney epithelial (NRK-52E, ATCC® CRL-6509™) cell lines by the MTT assay, which is widely used for the measurement of cell viability (48, 49). Briefly, the cells were seeded in 96-well plates at a density of 10^4 cells/well in 100 µL culture medium. Following 24-h incubation and attachment, the cells were treated with different concentrations of plant extracts and controls for 24 h. Dry methanolic extracts were dissolved in DMSO as a solvent to obtain appropriate stock solutions of the extracts. Dilution of stock extracts solutions was made in serum free medium yielding final extracts concentrations from 0.125 to 2 mg/mL. DMSO and 5-fluorouracil (5-FU) were used solvent and positive controls, respectively (32, 50). The concentration range used for 5-FU was 50 to 1000 µM. The yellow MTT dye was reduced by succinic dehydrogenase in the mitochondria of viable cells to purple formazan crystals. The absorbance was measured by a microplate reader (BioTek, USA) at 570 nm with a reference wavelength of 670 nm. The reduction of absorbance was evaluated the inhibition of enzyme activity observed in cells compared to untreated (negative control) cells. Then, the half maximal inhibitory concentration (IC_{50}) was expressed as the concentration of sample caused an inhibition of 50% in enzyme activities in cells as flows (48, 49). IC_{50} was calculated by using following Equation:

IC_{50} (%) = 100 - [mean absorbance of extract × 100)/mean absorbance of solvent control]

The results were generated from three independent experiments; each experiment was performed in triplicate.

Statistical analysis

Results were expressed as mean ± standard deviation. Statistical comparisons were performed with Student's *t*-test. Differences were considered significant at $p < 0.05$.

Results and Discussion

HPLC analysis

Table 1 shows the main components of the extracts of *Hypericum* species analyzed by HPLC and retention times, the equations and r^2 values obtained from calibration curves.

H. thymbrifolium, H. spectabile and *H. pseudolaeve* were enriched with chlorogenic acid. Our results are in an agreement with those reported in former studies in that the most common flavonoids present in *Hypericum* species under study are rutin, hyperoside, isoquercitrin, quercitrin, and quercetin (8, 10, 12-15).

Phytochemical composition of *Hypericum* species has been reported in various research works. The main compounds that gave a significant antioxidant activity from the ethanolic extract of *H. perforatum* were identified to be rutin and isoquercitrin as determined by HPLC, mass spectrometry, UV/Vis spectroscopy, and TLC (22, 23 and 51).

Total phenolic and flavonoid compounds

The yields, total phenolics and flavonoids of methanolic extracts obtained from aerial parts of *Hypericum* species are shown in Table 2. The amount of extractable compounds ranged from 149.1 to 213.5 mg/g DW. Among the three extracts, *H. spectabile* and *H. thymbrifolium* contained the highest amount of extractable compounds while the extract of *H. pseudolaeve* contained the lowest one.

The content of total phenols in extracts, ranged between 13.3 ± 1.70 and 23.1 ± 2.37 mg GAE/g DW. No significant differences ($p > 0.05$) were found between the amount of total phenolic compounds in *H. thymbrifolium* and *H. spectabile*, while the amount of phenolics in *H. pseudolaeve* were the lowest ($p < 0.05$).

Table 1. Chemical compounds of methanolic extracts of *Hypericum* species.

Compounds	Retention time (min)	Calibration equation values	Linear regression (r^2)	*H. spectabile* (yield%)	*H. pseudolaeve* (yield%)	*H. thymbrifolium* (yield%)
Pseudohypericin	4.86	y = 2.582269e + 007x + 1741.874	0.9998	0.0015 ± 0.0002	0.0131 ± 0.0004	0.0088 ± 0.0007
Hypericin	13.93	y = 6.03411e + 007x + 297.2292	0.9999	0.0070 ± 0.0001	0.0038 ± 0.0001	0.0044 ± 0.0001
Chlorogenic acid	4.33	y = 5110294x + 1490.398	0.9999	0.2357 ± 0.0269	0.3223 ± 0.0939	0.2957 ± 0.0603
Rutin	8.89	y = 1.383368e + 007x + 5188.182	0.9999	0.0083 ± 0.0004	0.1208 ± 0.0011	0.0100 ± 0.0006
Hyperoside	10.19	y = 2.849917e + 007x + 526.7023	0.9999	0.1138 ± 0.0065	0.2066 ± 0.0652	0.1681 ± 0.0381
Isoquercitrin	10.75	y = 1.671137e + 007x – 3712.788	0.9999	0.1387 ± 0.0126	0.1869 ± 0.0277	0.3038 ± 0.0661
Quercitrin	14.41	y = 1.205178e + 007 – 3518.974	0.9999	1.2863 ± 0.0554	0.2610 ± 0.0384	0.1553 ± 0.0121
Kaempferol	17.09	y = 5.183916e + 007x + 4373.856	0.9999	0.0081 ± 0.0008	0.0036 ± 0.0004	0.0007 ± 0.00003
Quercetin	17.84	y = 3.688175e + 007x + 18905.43	0.9999	0.0567 ± 0.0065	0.0592 ± 0.0052	0.0388 ± 0.0013
Amentoflavon	20.27	y = 2.207879e + 007x + 772.0972	0.9996	0.0030 ± 0.0001	0.0032 ± 0.0001	0.0027 ± 0.0001
Hyperforin	27.75	y = 6212343x	0.9997	0.0041 ± 0.0002	0.0023 ± 0.0002	Nd

*Values were the means of three replicates ± standard deviation, Nd: not determined.

Our results were consistent with the previous observation on the total phenolic content of some *Hypericum* species (24, 25 and 52). The flavonoid contents varied from 10.3 ± 0.23 to 22.4 ± 0.34 mg CE/g DW which is in accordance with the previously published data (53). The amount of flavonoids in *H. spectabile*, was higher than that of *H. thymbrifolium* and *H. pseudolaeve*.

Antioxidant activity

In the present study we evaluated the antioxidant activity of *Hypericum* species, measuring their ability of inhibiting LPO, reducing power, and radical scavenging activities. For comparison, Table 3 presents the results of the antioxidant activities, expressed as half maximal effective concentration (EC_{50}) and FRAP values.

All the extracts demonstrated the ability to inhibit LPO, which is in accordance with the previously published data for *Hypericum* species (22, 23, 25, 52, 54 and 55). The anti-LPO activities of *H. thymbrifolium* and *H. pseudolaeve* extracts were comparable ($p > 0.05$) and less effective than that of *H. spectabile*. However, none of the extracts were as effective LPO inhibitor as the reference antioxidant quercetin (0.06 ± 0.001 mg/mL).

H. thymbrifolium and *H. spectabile* did not differ in their DPPH radical scavenging activities ($p > 0.05$), which were higher than that of *H. pseudolaeve*. Our results were consistent with the previous observation that *Hypericum* species contain radical-scavenging agents that could directly react with and quench stable DPPH radicals (22, 25 and 55). Nonetheless, when compared to the the EC_{50} value obtained for the quercetin (0.034 ± 0.001 mg/mL), the DPPH scavenging activities of the extracts were

Table 2. Total extractable compounds (EC), total phenolic compounds (PC) (as gallic acid equivalents) and total flavonoids (as catechin equivalents) in the extracts.

Extracts	EC (mg/g DW)	PC (mg/g DW)	Flavonoid (mg/g DW)	PC/EC (%)
H. thymbrifolium	172.3	20.7 ± 2.1[a]	16.9 ± 0.51[a]	10.4
H. spectabile	213.5	23.1 ± 2.37[a]	22.4 ± 0.34[b]	10.8
H. pseudolaeve	149.1	13.3 ± 1.70[b]	10.3 ± 0.23[c]	8.9

Values were the means of three replicates ± standard deviation.
Values with different letters in the same column were significantly ($p < 0.05$) different.

Table 3. Antioxidant activities (EC_{50} values), AChE inhibitory and anti-inflammatory activities of the extracts.

Extracts	EC_{50} (mg/mL)[A]			FRAP value[B*] (mM Fe^{2+})	AChE* Inhibition (%)	COX-1* Inhibition (%)	COX-2* Inhibition (%)
	Anti-LPO	DPPH	Superoxide				
H. thymbrifolium	4.39 ± 0.08[a]	0.622 ± 0.051[a]	0.641 ± 0.069[a]	2.58 0.036[a]	63.41 ± 3.29[a]	71.77 ± 2.93[a]	64.14 ± 2.32[a]
H. spectabile	2.80 ± 0.28[b]	0.567 0.028[a]	0.430 ± 0.006[b]	2.66 ± 0.031[a]	59.49 ± 3.14[a]	77.04 1.55[a]	72.23 ± 5.41[a]
H. pseudolaeve	5.41 ± 0.55[a]	0.916 0.036[b]	1.730 ± 0.060[c]	2.21 ± 0.015[b]	49.37 ± 3.48[b]	43.27 5.44[b]	52.66 ± 3.03[b]
Quercetin	0.06 ± 0.001[c]	0.034 0.001[c]	0.513 ± 0.013[a, b]	2.84 ± 0.01[a, γ]			
Galantamine					89.86 ± 0.34[c, δ]		
Aspirin						73.53 3.57[a, ε]	

Values were the means of three replicates ± standard deviation. Values with different letters in the same column were significantly ($p < 0.05$) different.
[a]EC_{50} value: The effective concentration at which the antioxidant activity was 50%; DPPH and superoxide radicals were scavenged by 50%;
[b]Expressed as mM ferrous ions eqivalents. *Determined at 5 mg/mL. [γ]Determined at 1.25 mg/mL. [δ]Determined at 0.05 mg/mL. [ε]Determined at 0.5 mg/mL.
Antioxidant activities (EC_{50} values), AChE inhibitory and anti-inflammatory activities of the extracts.

Table 4. Cytotoxic potential of the extracts.

Extracts	IC_{50} values (mg/mL)	
	HeLa	NRK-52E
H. thymbrifolium	Na	Na
H. spectabile	Na	Na
H. pseudolaeve	1.218	0.964

Positive Control (5-FU): 48.012 µM for HeLa, 12.645 µM for NRK-52E.
Na: Non active.

found to be significantly lower ($p < 0.05$).

The superoxide radical scavenging activities of *H. thymbrifolium* and *H. spectabile* were comparable to that of quercetin (0.513 ± 0.013 mg/mL). In accordance with Hunt *et al.* observations, the results implied that *Hypericum* extracts are superoxide scavengers and their capacity to scavenge superoxide may contribute to their antioxidant activity (18).

In the FRAP assay, on the basis of the standard (Fe^{2+}), it was found that the extracts possess high reducing power (the range between 2.21 and 2.66 mM Fe^{2+}) at 5 mg/mL concentration. It was comparable ($p > 0.05$) to that of quercetin (2.84 ± 0.01 mM Fe^{2+}) at 1.25 mg/mL (Table 3). This observation was in an agreement with other reports (25, 53 and 55).

Our results showed that *H. spectabile* had a highest degree of potency in inhibiting LPO, demonstrated strongest reducing power and scavenging activity against the DPPH and superoxide radicals, indicating the highest antioxidant potential amongst the three extract under study. *H. thymbrifolium* demonstrated similar ability to that of *H. spectabile* to reduce ferric (III) iron to ferrous (II) iron and scavenge DPPH radical ($p > 0.05$) but was less effective LPO inhibitor together with *H. pseudolaeve*. The least effective antioxidant was *H. pseudolaeve*.

These findings were in agreement with our observation on phenolic contents of the extracts and seemed to suggest phenolics to be important contributors to the antioxidant activity. This result was in agreement with previous reports that the phenolic compounds contribute significantly to the antioxidant activity in different *Hypericum* species (19, 21-23, 40, 51, 53 and 56).

AChE inhibitory activity

Hypericum extracts were tested for their *in-vitro* AChE inhibitory activities using galantamine as a positive control. The results, expressed as percentage inhibitions, are summarized in Table 3.

The extracts exhibited moderate AChE inhibitory activities (49.37- 63.41%) at 5 mg/mL concentration. However, no plant extracts could have greater inhibitory ability than the positive control galantamine (89.86 ± 0.34%) at a concentration of 0.05 mg/mL (135 µM). Similar results were reported for *H. undulatum* by Ferreira *et al*. (57). Wszelaki *et al*. reported the lower (28%) AChE inhibition by methanolic extract of *H. perforatum* at a concentration of 400 µg/mL (33).

Anti-inflammatory activity

Methanol extracts of the aerial parts of *Hypericum* species under study were tested for their anti-inflammatory activity in comparison with aspirin used as the positive control. The ability of the extracts to inhibit COX-1 and COX-2 was determined by calculating percent inhibition of prostaglandin production (Table 3). It was found that all examined extracts exhibited inhibiting power against COX-1 and COX-2. The greatest anti-inflammatory effect was observed for *H. thymbrifolium* and *H. spectabile*. It was comparable to that of aspirin (73.5 ± 3.5%) at a concentration of 500 µg/mL.

Because of published evidence that flavonoids possess anti-inflammatory activity, the extract with the highest concentrations of flavonoids was expected to be the most anti-inflammatory (58). It can be explained with the different phytochemical composition of the extracts. *H. spectabile, H. thymbrifolium* and *H. pseudolaeve* extracts contained higher amount of chlorogenic acid. It was reported that chlorogenic acid contributed significantly to the anti-inflammatory activity of the *Hypericum* species (59). Chlorogenic acid represents a promising potential drug of natural anti-inflammatory property for the development of new drugs that may help to control oxidative stress and consequently the inflammatory response (60-62).

In-vitro cytotoxic activity

Hypericum species extracts were evaluated for their cytotoxic activities in HeLa and NRK-52E cell lines.

The extracts did not demonstrate significant cytotoxic activity against both HeLa and NRK-52E cell lines ($IC_{50} \leq 1.218$) (Table 4). Also, the responses of two cells to 5-FU were showed in Table 4. Our results were consistent with the previous observation that *Hypericum* species did not show significant cytotoxic activity against some tumor cell lines (23, 36, 37, 40, 63 and 64).

Conclusion

HPLC results showed that *Hypericum* species from Anatolia represent a good source of phenolic compounds. In particular, *H. spectabile* resulted endowed with high levels of quercitrin which may support its biological activities. In addition to this, the high content of quercitrin has a well known anti-inflammatory effect. As a conclusion, these results induce to further investigate the biological properties of *H. spectabile*.

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