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Evaluation of chemical composition, antioxidant and anti-acetylcholinesterase activities of *Hypericum neurocalycinum* and *Hypericum malatyanum*

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ABSTRACT

This study was conducted to evaluate the chemical composition, antioxidant and anti-acetylcholinesterase (anti-AChE) activities of methanolic extracts of *H. neurocalycinum* and *H. malatyanum*, two endemic species of the Turkish flora. 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. The extracts were tested *in vitro* for their antioxidant activities including, inhibition of lipid peroxidation in liposomes, induced by Fe^{3+}/ascorbate system, scavenging effect on DPPH• and superoxide anion radicals, and ferric ion reducing antioxidant power (FRAP). *H. neurocalycinum* demonstrated stronger antioxidant properties than *H. malatyanum* due to higher activity on scavenging DPPH• (EC_{50} = 2.49 ± 0.09 mg/mL) and superoxide anion radicals (EC_{50} = 0.613 ± 0.05 mg mg/mL), and inhibition of lipid peroxidation (EC_{50} = 2.49 ± 0.09 mg/mL). This difference in activity seems to be due to the presence of higher amounts of the antioxidant compounds (flavonoids) such as rutin, quercetin and kaempferol in *H. neurocalycinum* extract. The antioxidant activities of the extract may be attributed to their reducing capabilities. At 5 mg/mL *H. neurocalycinum* (FRAP value = 2.39 ± 0.039 mM Fe^{2+}) and *H. malatyanum* (FRAP value = 2.23 ± 0.013 mM Fe^{2+}) showed high ability to reduce Fe^{3+} to Fe^{2+}. The extracts were tested also for their *in vitro* AChE inhibitory activities. *H. neurocalycinum* inhibited 72.24 ± 0.39% of AChE activity at a concentration of 5 mg/mL. It was concluded that *H. neurocalycinum* is more effective AChE inhibitor and antioxidant than *H. malatyanum*.

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1. Introduction

Hypericum has 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 (Özkan et al., 2013). All members of the genus may be referred to as St. John's wort in the world. The genus *Hypericum* L. (Hypericaceae) has been used for the treatment of burns, wounds, bacterial and viral infections, hemorrhoids, diarrhea and ulcers in Turkish traditional medicine. Locally, it is traditionally used both externally (as a cream or oil extract) and internally (as a tea) with many therapeutic applications (Sezik et al., 2001; Kültür, 2007; Cirak et al., 2011). 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 (Greeson et al., 2001; Hunt et al., 2001; Smelcerovic et al., 2007; Demirkiran et al., 2013; Camas et al., 2014). Numerous antioxidant investigations have been carried out on *Hypericum* species (Conforti et al., 2007; Šavikin et al., 2007; Arruda et al., 2010; Hernandez et al., 2010; Altun et al., 2013; Zheleva-Dimitrova et al., 2013; Del Monte et al., 2015). The chemical composition of the *Hypericum* species is composed of naphthodianthrones (especially hypericin and pseudohypericin), acylphloroglucinol derivatives (especially hyperforin and adhyperforin), flavonoids (especially quercetin, quercitrin, hyperoside and biapigenin), tannins and volatile oils (Eroglu Ozkan and Mat, 2013). 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* species (Greeson et al., 2001; Conforti et al., 2002; Conforti et al., 2005; Conforti et al., 2007; Smelcerovic et al., 2007; Cirak et al., 2013; Demirkiran et al., 2013; Camas et al., 2014).

Alzheimer's disease (AD) is a neurodegenerative disorder that is the most common cause of dementia in the elderly (Mukherjee et al., 2007). Acetylcholine is involved in the signal transfer in the synapses. Acetylcholinesterase (AChE), is a key enzyme in the nervous system,

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terminates the action of acetylcholine at the post-synaptic membrane in the neuromuscular junction (Ferreira et al., 2006). Enhancing acetylcholine level in the brain through the use of cholinesterase inhibitors is the first treatment strategy in AD. Some licensed cholinesterase inhibitors are naturally derived (galantamine and rivastigmine) and already used for the treatment of dementia, primarily AD (Howes and Perry, 2011).

Oxidative stress is defined as an imbalance between the concentration of oxidant (reactive oxygen species-ROS) and antioxidative defense mechanisms in favor of the oxidants. It has been demonstrated that oxidative stress plays an important role in AD (Gibson and Huang, 2005). In AD, amyloid β peptide (Aβ) accumulates in plaques in the brain. ROS is involved in Aβ-induced neurotoxicity and the progression of AD (Lu et al., 2009). Antioxidants scavenge ROS and inhibit formation of free radical intermediates, thus may be useful in treatment of AD (Dumont and Beal, 2011).

The aim of the present study was to investigate the chemical profiles, antioxidant and (AChE) inhibitory potential of the extracts from two endemic *Hypericum* species (*Hypericum neurocalycinum* Boiss. & Heldr. and *Hypericum malatyanum* Peşmen) of the Turkish flora. Our research is the first report to study the phytochemical profiles and biological activities in these species.

2. Material and methods

2.1. 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 and quercetinwere purchased from Fluka (Buchs, Switzerland). Phenazine methosulfate (PMS), 2,2-diphenyl-1-picryl-hydrazyl (DPPH), gallic acid, ascorbic acid, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide (ATChI), AChE, and 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).

2.2. Plant material

During the field investigations conducted in June 2010, specimens of flowering aerial parts of *H. neurocalycinum* and *H. malatyanum* were gathered from their natural habitats of Konya (located in the Inner Anatolia Region of Turkey) and Malatya (located in the East Anatolia Region of Turkey). 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 93195 and 93196, respectively).

2.3. 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 obtained 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 (Çirak et al., 2006; Mahavorasirikul et al., 2010). The extracts prepared with this procedure were used in the HPLC analysis and biological activity studies.

2.4. HPLC analysis

2.4.1. 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.

2.4.2. Preparation of the samples

The crude methanol extract was dissolved in mixture of methanol/water (8:2, v/v) (Özkan et al., 2013). All samples were filtered through a 0.45-μm filter into a vial for HPLC analysis. Each sample was prepared and injected three times.

2.4.3. 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 i.d., 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

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

Compounds	Retention time (min)	Calibration equation values	Linear regression (r^2)	*H. neurocalycinum* (yield, %)	*H. malatyanum* (yield, %)
Pseudohypericin	4.86	y = 2.582269e + 007x + 1741.874	0.9998	0.00016 ± 0.00001	**0.00930 ± 0.00060**
Hypericin	13.93	y = 6.03411e + 007x + 297.2292	0.9999	**0.00322 ± 0.00010**	0.00304 ± 0.00003
Chlorogenic acid	4.33	y = 5,110,294x + 1490.398	0.9999	**0.26033 ± 0.02752**	0.01072 ± 0.00072
Rutin	8.89	y = 1.383368e + 007 + 5188.182	0.9999	**0.03299 ± 0.00111**	0.01967 ± 0.00200
Hyperoside	10.19	y = 2.849917e + 007x + 526.7023	0.9999	0.30434 ± 0.05273	**0.58745 ± 0.05183**
Isoquercitrin	10.75	y = 1.671137e + 007x − 3712.788	0.9999	0.12173 ± 0.08600	**0.19994 ± 0.01220**
Quercitrin	14.41	y = 1.205178e + 007 − 3518.974	0.9999	Nd	**0.00770 ± 0.00071**
Kaempferol	17.09	y = 5.183916e + 007x + 4373.856	0.9999	**0.00482 ± 0.00019**	0.00188 ± 0.00011
Quercetin	17.84	y = 3.688175e + 007 + 18,905.43	0.9999	**0.13809 ± 0.01178**	0.09261 ± 0.00821
Amentoflavon	20.27	y = 2.207879e + 007 + 772.0972	0.9996	0.00144 ± 0.00004	**0.00720 ± 0.00047**
Hyperforin	27.75	y = 6,212,343x	0.9997	Nd	**0.00120 ± 0.00019**

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