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Screening of *in vitro* antimicrobial and antioxidant activity of nine *Hypericum* species from the Balkans

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Abstract

The *in vitro* antimicrobial and antioxidant activities of the crude methanol extracts of nine *Hypericum* species (*H. barbatum* Jacq., *H. hirsutum* L., *H. linarioides* Bosse, *H. maculatum* Crantz, *H. olympicum* L., *H. perforatum* L., *H. richeri* Vill., *H. rumeliacum* Boiss. and *H. tetrapterum* Fries) growing spontaneously in the Balkans were screened using a disk diffusion assay against a panel of standardized bacteria and fungi and a phosphomolybdenum method, respectively. The most active extract against all microbial strains was that of *Hypericum hirsutum* (collected at Vranjska Banja, South Serbia), which showed remarkable antimicrobial activity at a dose of 5 μg per disk and a diameter of growth inhibition zone reaching values more than two times larger than that of standard antibiotics. The antimicrobial activity of *H. barbatum*, *H. hirsutum*, *H. olympicum*, *H. richeri* and *H. tetrapterum* has not hitherto been published. All the extracts possess a very broad spectrum of strong antimicrobial activity. All the investigated species, except *H. perforatum*, have been tested for the first time in an antioxidant capacity assay. The results are correlated and discussed with respect to the until now known constituents of the investigated species.
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Keywords: *Hypericum*; Hypericaceae; Methanol extract; Antimicrobial activity; Antioxidant activity

1. Introduction

Renewed interest in plant antimicrobials has emerged during the last 20 years (Cowan, 1999; De Smet, 1997; Kelmanson, Jäger, & Van Staden, 2000; Srinivasan, Nathan, Suresh, & Perumalsamy, 2001), probably due to the increasing development of drug resistance to human pathogenic organisms, as well as the appearance of undesirable side effects of certain antibiotics and the emergence of previously uncommon infections (Davies, 1994; Marchese & Schito, 2001; Poole, 2001).

Plant species of the genus *Hypericum* are well known for their use in traditional medicine, due to the therapeutic efficacy of its many different species. One of the most important and most recognized species of the genus is *Hypericum perforatum* L., St. John's wort, which has been used in herbal medicine, externally for the treatment of skin wounds, eczema and burns and, internally, for disorders of the central nervous system, the alimentary tract and other purposes (Barnes, Anderson, & Phillipson, 2001; Bombardelli & Morazzoni, 1995). A review on the antimicrobial activity of *H. perforatum* extracts was published recently (Reichling, Weseler, & Saller, 2001). Several studies have revealed antidepressant, antiviral, wound healing, antioxidant and antimicrobial activity of various extracts of *H. perforatum* (Barnes et al., 2001; Butterwerck, Jürgenliemk, Nahrstedt, & Winterhoff, 2000; Sakar & Tamer, 1990). Other species of the genus *Hypericum* have also been found to exert antimicrobial and wound healing

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effects (Decosterd, Hoffmann, Kyburz, Bray, & Jostettmann, 1991; Dulger & Gonuz, 2005; Jayasuriyab, Clark, & McChesney, 1991; Mukherjee, Verpoorte, & Suresh, 2000; Rabanal, Arias, Prado, Hernández-Pérez, & Sánchez-Mateo, 2002; Rocha et al., 1995). The indigenous *Hypericum* species from the Balkans have received little attention until now, except for studies of the essential oils, which have also been frequently tested for their antimicrobial properties (Cakir, Kordali, Kilic, & Kaya, 2005; Couladis, Chinou, Tzakou, & Petrakis, 2003; Gudzic, Djordjevic, Palic, & Stojanovic, 2001, 2002). Usually mistaken to be St. John's wort, the other indigenous *Hypericum* species, including the ones studied in this work, therefore have the same relevance and utilization in the diet of Balkan people as the true *H. perforatum*.

There is an increasing interest in natural antioxidants, namely phenols, present in medicinal and dietary plants, that might help prevent oxidative damage (Gardner, White, McPhail, & Duthie, 2000; Halliwell, 1999; Zheng & Wang, 2001). Oxidative stress can play an important role in the development of neurodegradative disorders, such as Alzheimer's disease (Behl & Mosmann, 2002) and is implicated in the development of chronic diseases, such as cancer, arteriosclerosis and rheumatism (Halliwell, 1999).

A survey of the recent literature showed rather scarce data on the antioxidant activity of *Hypericum* species (Conforti, Statti, Tundis, Menichini, & Houghton, 2002), compared to other investigated biological properties, and these mainly dealt with the activity of *H. perforatum* (Cakir et al., 2003; Hunt, Lester, Lester, & Tackett, 2001; Silva, Ferreres, Malva, & Dias, 2005; Zou, Lu, & Wei, 2004). A study on the antioxidative activity of 21 phloroglucinol derivatives isolated from *Hypericum* species has been undertaken (Heilmann, Winkelmann, & Sticher, 2003).

In this work, we report the antimicrobial and antioxidant activities of methanol extracts of nine species of this genus (*Hypericum barbatum* Jacq., *Hypericum hirsutum* L., *Hypericum linarioides* Bosse, *Hypericum maculatum* Crantz, *Hypericum olympicum* L., *H. perforatum* L., *Hypericum richeri* Vill., *Hypericum rumeliacum* Boiss. and *Hypericum tetrapterum* Fries). This represents the first report on the antimicrobial activity of the extracts of most of the investigated species, except for *Hypericum perforatum* (the subject of many studies), as well as *H. rumeliacum*, *H. linarioides* and *H. maculatum* (essential oils tested for antimicrobial (Couladis et al., 2003; Gudzic, Djokovic, Vajs, Palic, & Stojanovic, 2002) and antifungal activity (Cakir et al., 2005), respectively). All the investigated species, except *H. perforatum*, have been tested for the first time in an antioxidant capacity assay.

2. Material and methods

2.1. Chemicals and reagents

Analytical grade ammonium molybdate, dimethyl sulfoxide, methanol, sulfuric acid, sodium phosphate and α-tocopherol acetate were obtained from Merck (Darmstadt, Germany).

2.2. Plant material

Table 1 contains the data concerning the identity of the *Hypericum* species under study, voucher numbers of the deposited herbarium specimens (Herbarium Moesicum Doljevac, Serbia and Montenegro), site and date of collection, as well as their taxonomic placement within sections of the genus *Hypericum* (Robson, 1977). All the plant species samples were collected at bloom stage.

2.3. Preparation of plant extracts

The dried and powdered plant material (2 g of aerial parts if not specified otherwise in Table 1) were extracted at room temperature with methanol (20 ml) by maceration (3×24 h), yielding total methanol crude extracts (TMCE) which were evaporated to dryness *in vacuo* at 45 °C (to avoid any loss or degradation of plant constituents). The yields (%, w/w dry plant material) of dry extracts are presented in Table 1. The dried extracts were dissolved in methanol to a final concentration of 100 μg/ml and used as such for the antimicrobial and antioxidant testing.

2.4. Test microorganisms

The *in vitro* antimicrobial activities of the extracts A–I were tested against a panel of laboratory control strains belonging to the American Type Culture Collection Maryland, USA: Gram-positive:*Staphylococcus aureus* (ATCC 6538), Gram-negative: *Klebsiella pneumoniae* (ATCC 10031), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella enteritidis* (ATCC 13076), and fungal organisms *Aspergillus niger* (ATCC 16404) and *Candida albicans* (ATCC 10231), except for the Gram-negative bacteria *Escherichia coli* 95, which was obtained from the Institute of Immunology and Virology "Torlak", Belgrade, Serbia and Montenegro.

2.5. Antimicrobial assay

A disk diffusion method, according to NCCLS (1997), was employed for the determination of the antimicrobial activity of the extracts (TMCE). The following nutritive media were used: Antibiotic Medium 1 (Difco Laboratories, Detroit Michigan USA) for growing Gram-positive and Gram-negative bacteria and Tripton soy agar (TSA – Torlak, Belgrade) for *Candida albicans* and *Aspergillus niger*. Nutritive media were prepared according to the instructions of the manufacturer. All agar plates were prepared in 90 mm Petri dishes with 22 ml of agar, giving a final depth of 4 mm. One-hundred microlitres of a suspension of the tested microorganisms (10^8 cells per ml) were

Table 1
Relevant data on the studied *Hypericum* species from the Balkans and the yields of the crude dry methanol extracts

Section	Symbol	Plant species	Voucher number (HMD)	Collection period	Locality	Extract yield (%, w/w)
Drosocarpium Spach	A1	*H. barbatum* Jacq.	704	June 2003	Besna kobila, South east Serbia	11.3
	A2	*H. barbatum* Jacq.	705	July 2003	Rtanj, East Serbia	11.1
	A3	*H. barbatum* Jacq.	706	July 2003	Galičica, West F.Y.R. Macedonia	8.7
	A4	*H. barbatum* Jacq.	723	August 2004	Suva planina, East Serbia	15.3
	B1	*H. richeri* Vill.	715	August 2003	Suva planina, East Serbia	5.6
	C1	*H. rumeliacum* Boiss.	716	July 2003	Seličevica, East Serbia	3.6
	C2	*H. rumeliacum* Boiss.	717	July 2003	Rujan planina, South Serbia	5.6
	C3	*H. rumeliacum* Boiss.	718	July 2003	Sićevačka klisura, East Serbia	1.7
	C4	*H. rumeliacum* Boiss.	719	July 2003	Rudina planina, South east Serbia	4.3
	C5	*H. rumeliacum* Boiss.	729	May 2004	Sićevačka klisura, East Serbia	4.5
	C6	*H. rumeliacum* Boiss.	730	July 2004	Rudina planina, South east Serbia	4.4
Hypericum	D1	*H. maculatum* Crantz	710	June 2003	Besna kobila, South east Serbia	13.1
	D2	*H. maculatum* Crantz	711	July 2003	Stara planina, East Serbia	16.8
	D3	*H. maculatum* Crantz	712	July 2003	Vlasinsko jezero, South east Serbia	18.5
	D4	*H. maculatum* Crantz	726	August 2004	Suva planina, East Serbia	19.3
	E1	*H. perforatum* L.	714	July 2003	Rujan planina, South Serbia	12.7
	E2	*H. perforatum* L. (flowers)	728	June 2004	Pašina česma, South east Serbia	17.1
	E3	*H. perforatum* L. (leaves)	728	June 2004	Pašina česma, South east Serbia	13.5
	E4	*H. perforatum* L. (stems)	728	June 2004	Pašina česma, South east Serbia	0.8
	F1	*H. tetrapterum* Fries	720	July 2003	Leska, East Serbia	12.5
	F2	*H. tetrapterum* Fries	721	August 2003	Osogovske planine, East F.Y.R. Macedonia	16.1
	F3	*H. tetrapterum* Fries	722	August 2003	Beljanica, East Serbia	14.4
	F4	*H. tetrapterum* Fries	731	July 2004	Rudina planina, South east Serbia	10.3
Olympia (Spach) Nyman	G1	*H. olympicum* L.	713	July 2003	Rujan planina, South Serbia	13.1
	G2	*H. olympicum* L.	727	July 2004	Kumanovo, North F.Y.R. Macedonia	22.4
Taeniocarpium Jaub. et Spach	H1	*H. hirsutum* L.	707	July 2003	Vranjska Banja, South Serbia	15.5
	H2	*H. hirsutum* L.	708	August 2003	Suva planina, East Serbia	13.0
	H3	*H. hirsutum* L.	724	August 2004	Suva planina, East Serbia	12.0
	I1	*H. linarioides* Bosse	709	August 2003	Suva planina, East Serbia	31.4
	I2	*H. linarioides* Bosse	725	August 2004	Suva planina, East Serbia	35.3

spread on the solid media plates. Sterile filter paper disks (''Antibiotica Test Blattchen'', Schleicher and Schuell, Dassel, Germany, 12.7 mm in diameter) were impregnated with 50 μl of the TMCE extracts (all extracts were filter-sterilized using a 0.45 μm membrane filter) and placed on inoculated plates. These plates, after standing at 4 °C for 2 h, were incubated at 37 °C for 24 h for bacteria and at 30 °C for 48 h for the fungi. Standard disks of Tetracycline, Gentamicin, Ampicillin and Nystatine (origin – Institute of Immunology and Virology "Torlak", 30 μg of the active component, diameter 6 mm) were used individually as positive controls, while the disks imbued with 50 μl of pure methanol were used as a negative control. The diameters of the inhibition zones were measured in millimeters (to the nearest 0.1 mm) using a "Fisher-Lilly Antibiotic Zone Reader" (Fisher Scientific Co., USA). Each test was performed in quintuplicate and repeated three times.

2.6. Determination of the total antioxidant capacity

The total antioxidant capacity of the crude methanol extracts (TMCE) of nine *Hypericum* species (30 samples) was evaluated by the method of Prieto, Pineda, and Aguilar (1999). The antioxidant capacity of the extracts was measured spectrophotometrically using a phosphomolybdenum method, based on the reduction of Mo(VI) to Mo(V) by the sample analyte and the subsequent formation of green phosphate/Mo(V) compounds with a maximum absorption at $\lambda = 695$ nm. A 0.1 ml aliquot of sample solution (100 μg/ml) was combined in an Eppendorf tube with 1 ml of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95 °C for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm, against

a blank solution, using a Perkin–Elmer Lambda 15 UV–vis spectrophotometer. A typical blank solution contained 1 ml of reagent solution and the appropriate volume of methanol used for the dissolution of the samples and it was incubated under the same conditions as the other samples. Stock solutions of α-tocopherol acetate were prepared in methanol just prior to use. Exact concentrations were determined spectrophotometrically on the basis of the absorption coefficients from the literature. Since the samples were of unknown composition, the total antioxidant capacity was expressed as equivalents of α-tocopherol acetate (μmol/g of extract).

2.7. Statistical analysis

In order to evaluate statistically any significant differences among mean values, a one-way ANOVA test was used. In all tests the significance level at which we evaluated critical values differences was 5%. The mean values followed by different superscripts within each column of Tables 2 and 3 indicate that they were significantly different at that probability level.

3. Results and discussion

The antimicrobial activity of the crude methanol extracts (TMCEs) of nine *Hypericum* species from the Balkans was studied using a disk diffusion assay recommended by NCCLS (1997) against seven microorganisms. The obtained results are presented in Table 2. Our results show remarkable antimicrobial activity for the methanol extract of *H. hirsutum* (especially the H1 TMCE) against all microorganisms tested, both bacteria and fungi, with inhibition zones in some cases reaching more than twice the value of the standard antibiotics used as positive controls (at 30 μg per disk). Further, the TMCEs of *H. linarioides* (I1 and I2), *H. tetrapterum* (F2 and F4), *H. olympicum* (G1) and *H. rumeliacum* (C5) showed a broad spectrum of very strong antimicrobial activity. The diameters of growth inhibition zones ranged from 14 to 46 mm (rounded to integers,

Table 2
The antimicrobial activity (diameters of growth inhibition zones[a]) of the crude methanol extracts of nine *Hypericum* species from the Balkans

Extract/Microorganism	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*S. aureus*	*S. enteritidis*	*A. niger*	*C. albicans*
H. barbatum Jacq. A1	20.66 ± 0.09^{ab}	26.07 ± 0.57^{a}	18.08 ± 0.48^{a}	27.07 ± 0.04^{a}	30.06 ± 0.67^{a}	17.38 ± 0.61^{a}	21.29 ± 0.08^{a}
H. barbatum Jacq. A2	26.73 ± 0.28^{b}	28.30 ± 0.98^{bc}	30.03 ± 0.92^{b}	22.73 ± 0.91^{b}	25.09 ± 0.62^{b}	17.22 ± 0.10^{a}	25.92 ± 0.37^{b}
H. barbatum Jacq. A3	27.64 ± 0.10^{c}	26.01 ± 0.02^{a}	30.97 ± 0.07^{c}	20.22 ± 0.37^{c}	23.28 ± 0.32^{c}	25.90 ± 1.63^{bc}	22.00 ± 0.64^{c}
H. barbatum Jacq. A4	23.51 ± 0.94^{def}	30.76 ± 0.49^{d}	17.25 ± 0.25^{d}	30.02 ± 0.98^{d}	25.03 ± 0.94^{b}	27.11 ± 0.27^{d}	20.07 ± 0.72^{de}
H. richeri Vill. B1	18.77 ± 0.96^{g}	29.73 ± 0.52^{e}	22.02 ± 0.11^{e}	19.22 ± 0.61^{e}	27.06 ± 0.94^{d}	24.37 ± 1.09^{e}	18.94 ± 0.61^{f}
H. rumeliacum Boiss. C1	15.47 ± 0.82^{h}	14.61 ± 0.04^{f}	15.37 ± 0.95^{f}	14.27 ± 0.38^{f}	20.05 ± 0.82^{e}	15.29 ± 0.62^{f}	16.37 ± 0.10^{g}
H. rumeliacum Boiss. C2	24.96 ± 1.02^{ijk}	14.93 ± 0.11^{g}	25.26 ± 0.37^{g}	30.08 ± 0.09^{d}	20.36 ± 0.20^{e}	20.08 ± 0.28^{ghi}	17.39 ± 0.84^{h}
H. rumeliacum Boiss. C3	14.41 ± 0.11^{l}	14.52 ± 0.79^{fghi}	14.10 ± 0.58^{h}	14.03 ± 0.73^{f}	14.84 ± 0.13^{f}	16.94 ± 0.71^{a}	15.04 ± 0.15^{i}
H. rumeliacum Boiss. C4	23.86 ± 0.41^{em}	14.40 ± 0.92^{fhij}	18.08 ± 0.64^{a}	16.81 ± 0.74^{g}	14.37 ± 0.34^{g}	16.38 ± 0.31^{j}	16.29 ± 0.96^{g}
H. rumeliacum Boiss. C5	24.92 ± 0.07^{i}	36.99 ± 0.31^{k}	24.00 ± 0.44^{i}	28.36 ± 0.55^{h}	23.07 ± 1.20^{c}	15.37 ± 0.91^{f}	25.11 ± 0.64^{j}
H. rumeliacum Boiss. C6	15.96 ± 0.06^{n}	14.05 ± 0.07^{j}	29.03 ± 0.09^{j}	27.24 ± 1.03^{a}	19.19 ± 0.89^{h}	18.19 ± 0.92^{kl}	15.00 ± 0.67^{i}
H. maculatum Crantz D1	30.52 ± 0.40^{o}	28.63 ± 0.10^{c}	23.22 ± 1.16^{k}	25.25 ± 0.23^{i}	24.76 ± 0.31^{b}	23.52 ± 0.35^{m}	24.23 ± 0.67^{k}
H. maculatum Crantz D2	17.92 ± 0.38^{p}	16.08 ± 0.08^{l}	30.08 ± 0.91^{b}	23.29 ± 0.17^{j}	20.82 ± 0.37^{i}	20.02 ± 0.31^{gn}	20.29 ± 0.37^{e}
H. maculatum Crantz D3	23.24 ± 0.19^{d}	40.50 ± 0.66^{m}	32.31 ± 0.07^{l}	19.08 ± 0.69^{e}	20.33 ± 0.64^{e}	19.95 ± 0.76^{ghi}	20.69 ± 0.37^{l}
H. maculatum Crantz D4	34.92 ± 0.70^{q}	35.97 ± 0.33^{n}	36.54 ± 2.04^{m}	18.31 ± 0.21^{k}	32.33 ± 0.26^{j}	20.31 ± 0.73^{ino}	21.22 ± 0.37^{am}
H. perforatum L. E1	20.50 ± 0.53^{ar}	16.31 ± 0.94^{lo}	18.24 ± 0.32^{a}	21.06 ± 0.73^{l}	18.22 ± 0.15^{k}	19.36 ± 0.07^{p}	20.04 ± 0.35^{de}
H. perforatum L. E2	24.15 ± 0.66^{ms}	28.15 ± 0.21^{b}	30.34 ± 0.10^{b}	29.11 ± 0.30^{m}	18.27 ± 0.21^{k}	20.29 ± 0.33^{hio}	29.57 ± 0.26^{n}
H. perforatum L. E3	14.23 ± 0.73^{l}	17.05 ± 0.47^{p}	15.37 ± 0.76^{f}	22.61 ± 0.72^{b}	18.00 ± 0.83^{kl}	24.11 ± 0.64^{e}	17.31 ± 0.77^{h}
H. perforatum L. E4	22.11 ± 0.07^{t}	14.72 ± 0.14^{i}	15.23 ± 0.98^{f}	14.99 ± 0.28^{n}	14.55 ± 0.31^{g}	22.02 ± 0.06^{q}	16.31 ± 0.60^{g}
H. tetrapterum Fries F1	25.12 ± 0.08^{k}	24.34 ± 0.09^{q}	23.88 ± 0.76^{ik}	28.14 ± 0.37^{o}	20.07 ± 0.39^{e}	18.16 ± 0.22^{l}	24.27 ± 0.87^{k}
H. tetrapterum Fries F2	22.58 ± 0.17^{u}	31.30 ± 0.87^{r}	32.12 ± 0.07^{n}	34.31 ± 0.81^{p}	30.28 ± 0.94^{a}	18.08 ± 0.67^{kl}	28.21 ± 0.38^{o}
H. tetrapterum Fries F3	20.33 ± 0.59^{rv}	25.77 ± 1.16^{as}	33.06 ± 0.54^{o}	28.91 ± 0.34^{m}	28.60 ± 1.28^{mn}	21.23 ± 0.39^{r}	25.06 ± 0.68^{j}
H. tetrapterum Fries F4	25.07 ± 0.51^{ik}	24.08 ± 0.05^{t}	32.76 ± 0.26^{o}	25.00 ± 0.13^{q}	30.11 ± 0.92^{a}	17.73 ± 0.16^{k}	25.42 ± 1.23^{bj}
H. olympicum L. G1	24.60 ± 0.58^{js}	30.38 ± 0.65^{d}	30.25 ± 0.61^{b}	21.28 ± 0.46^{l}	28.05 ± 0.51^{m}	26.03 ± 0.88^{b}	29.33 ± 0.14^{p}
H. olympicum L. G2	20.17 ± 0.04^{v}	17.40 ± 0.71^{p}	24.37 ± 0.51^{p}	25.22 ± 0.38^{i}	24.03 ± 0.16^{o}	25.34 ± 0.63^{c}	20.01 ± 0.13^{d}
H. hirsutum L. H1	40.97 ± 0.84^{w}	41.72 ± 0.59^{u}	40.11 ± 0.62^{q}	30.97 ± 0.12^{r}	45.99 ± 0.92^{p}	33.30 ± 0.96^{s}	36.09 ± 0.31^{q}
H. hirsutum L. H2	26.21 ± 0.92^{x}	25.74 ± 0.12^{s}	18.86 ± 0.34^{r}	26.37 ± 0.26^{s}	17.91 ± 0.02^{l}	18.18 ± 0.21^{l}	18.38 ± 0.94^{f}
H. hirsutum L. H3	28.53 ± 0.72^{y}	20.70 ± 0.29^{v}	19.06 ± 0.21^{r}	23.90 ± 0.34^{t}	29.08 ± 0.27^{n}	20.91 ± 0.97^{or}	21.36 ± 0.72^{am}
H. linarioides Bosse I1	31.55 ± 0.23^{z}	32.85 ± 0.44^{w}	37.28 ± 0.68^{m}	36.04 ± 0.61^{o}	32.22 ± 0.38^{j}	25.09 ± 0.05^{c}	24.23 ± 0.47^{k}
H. linarioides Bosse I2	$22.30 \pm 0.25^{\alpha}$	35.99 ± 0.08^{n}	38.29 ± 0.67^{s}	35.29 ± 0.35^{u}	33.23 ± 0.88^{q}	22.13 ± 0.09^{t}	20.94 ± 0.53^{lm}
Tetracycline	$30.64 \pm 0.53^{\beta}$	30.30 ± 0.77^{d}	29.31 ± 0.63^{j}	30.66 ± 0.42^{v}	31.30 ± 0.73^{r}	nt	nt
Gentamicin	24.22 ± 0.99^{mfsj}	16.52 ± 0.62^{o}	19.84 ± 0.87^{t}	19.19 ± 1.23^{e}	20.22 ± 0.35^{e}	nt	nt
Ampicillin	$12.18 \pm 0.61^{\gamma}$	14.11 ± 0.09^{hj}	na[u]	na[w]	11.23 ± 0.14^{s}	nt	nt
Nystatine	nt	nt	nt	nt	nt	18.99 ± 0.27^{u}	17.21 ± 0.34^{j}

na, not active; nt, not tested.
[a] Mean values ± SD (in mm) of 15 experiments, including the diameter of the disc (12.7 mm).
[b] The mean values followed by different superscripts within each column indicate that they were significantly different at a probability level of 0.05 according to ANOVA test.

Table 3
The total antioxidant capacity of the crude methanol extracts of *Hypericum* species from the Balkans expressed as equivalents of α-tocopherol acetate (μmol/g of extract)[a]

Extract	Antioxidant activity[a]
H. barbatum Jacq. A1	6060 ± 22[a]
H. barbatum Jacq. A2	60900 ± 46[b]
H. barbatum Jacq. A3	12600 ± 102[c]
H. barbatum Jacq. A4	2520 ± 46[d]
H. richeri Vill. B1	7470 ± 79[e]
H. rumeliacum Boiss. C1	12300 ± 127[f]
H. rumeliacum Boiss. C2	2050 ± 90[g]
H. rumeliacum Boiss. C3	37300 ± 111[h]
H. rumeliacum Boiss. C4	5420 ± 25[i]
H. rumeliacum Boiss. C5	3770 ± 59[j]
H. rumeliacum Boiss. C6	4050 ± 72[k]
H. maculatum Crantz D1	26300 ± 73[l]
H. maculatum Crantz D2	4070 ± 41[k]
H. maculatum Crantz D3	3730 ± 40[j]
H. maculatum Crantz D4	2290 ± 40[m]
H. perforatum L. E1	1560 ± 95[n]
H. perforatum L. E2	73100 ± 49[o]
H. perforatum L. E3	5430 ± 55[i]
H. perforatum L. E4	3630 ± 83[p]
H. tetrapterum Fries F1	8720 ± 98[q]
H. tetrapterum Fries F2	2580 ± 73[d]
H. tetrapterum Fries F3	4810 ± 55[r]
H. tetrapterum Fries F4	16000 ± 60[s]
H. olympicum L. G1	4290 ± 30[t]
H. olympicum L. G2	540 ± 11[u]
H. hirsutum L. H1	3370 ± 22[v]
H. hirsutum L. H2	15700 ± 40[w]
H. hirsutum L. H3	5210 ± 84[x]
H. linarioides Bosse I1	5600 ± 83[y]
H. linarioides Bosse I2	1400 ± 30[z]

[a] The mean values (±SD of five experiments) followed by different superscripts within each column indicate that they were significantly different at a probability level of 0.05 according to ANOVA test.

including the diameter of the disk, 12.7 mm), with the highest inhibition zone values observed against the medically important pathogens *K. pneumoniae* (42 mm) and *S. enteritidis* (46 mm). All the microorganisms were completely unsusceptible to control disks imbued with methanol.

From the results obtained, it appears that the antibacterial action of the extracts is more pronounced on Gram-negative than on Gram-positive (*S. aureus*) bacteria in most cases or is even equal. These findings do not correlate with the observations of previous screenings of medicinal plants for antimicrobial activity, where most of the active plant extracts showed activity against Gram-positive strains only (Ali, Jülich, Kusnick, & Lindequist, 2001; Herrera, Pérez, Martín-Herrera, López-García, & Rabanal, 1996; Kelmanson et al., 2000).

On the other hand, the relatively lower values for the inhibition zones for *H. rumeliacum* extracts (C1, C3, C6) confirmed their bacteriostatic activity and, although these values are lower compared to those of usual antibiotics, these results are of interest since they have been obtained with crude extracts that are active at 5 μg per disk in a diffusion assay and could be considered to have a good potency level (Rios, Recio, & Villar, 1988).

Two previous screening studies of Brazilian *Hypericum* species (Dall'Agnol et al., 2003; Fenner et al., 2005) showed that all of the investigated species contained substances that delay *C. albicans* growth and that this could explain the traditional use of the plants to treat mouth wounds, including aphthas (Correa, 1984; Mentz, Lutzemberger, & Schenkel, 1997). However, since in this study the two fungal organisms (*C. albicans* and *A. niger*) were among the least susceptible strains tested, no such observation could be made.

The results show considerable variation in the TMCE yields and antimicrobial activity with respect to the site and date of collection (TMCE of *H. rumeliacum* C1 and C5 and *H. barbatum* A1 and A2 can serve as examples).

A rough generalization, based on the taxonomic classification of the investigated *Hypericum* species (Robson, 1977), can be made that the antifungal activity of the TMCEs decreases in the following order of sections: *Taeniocarpium* Jaub. and Spach > *Olympia* (Spach) Nyman ≈ *Hypericum* > *Drosocarpium* Spach. However, species belonging to all of the mentioned sections show comparable bactericidal activity (the only observable separation being that of a significant lead of section *Taeniocarpium*).

According to the results of this screening, the *H. perforatum* extract does not stand out as the most active. Although it is the most recognized worldwide for its antidepressive activity, and the most examined, it is in fact among the least active of the TMCEs in the current antimicrobial assay.

With regard to the components responsible for the strong broad spectrum of antimicrobial activity shown, several compounds of distinct nature must be acting as antimicrobial agents in these plants. This is not unexpected, since in the majority of *Hypericum* species studied so far there is a large variety of active principles, including naphthodianthrones, flavonoids, xanthones, tannins, essential oils and phloroglucinols (Bombardelli & Morazzoni, 1995; Ishiguro et al., 1990; Jayasuriyab et al., 1991; Pistelli et al., 2000; Rocha et al., 1995; Sakar & Tamer, 1990). Since the antimicrobial activity in other species of this genus has been found to be closely related to the levels of flavonoids and phloroglucinol derivatives (Bombardelli & Morazzoni, 1995; Ishiguro et al., 1990; Jayasuriyab et al., 1991; Rocha et al., 1995), it is reasonable to assume that these compounds are responsible for the antimicrobial activities reported here. Spectrophotometric analysis of the flavonoid contents, undertaken by Males, Plazibat, Vundac, Zuntar, and Pilepic (2004), indicated that *H. perforatum* is richer in flavonoids than *H. hirsutum*; this is again contrary to the observed activity. The other species (six) except *H. linarioides* have also been reported to contain flavonoids and tannins (Akhtardzhiev, Nakov, & Tsendov, 1973; Corovic, Stjepanovic, Nikolic, Pavlovic, & Zivanovic, 1965; Kitanov, 1979; Kitanov & Nikolov, 1991; Maggi, Ferretti, Pocceschi, Menghini, & Ricciutelli, 2004). Results suggest that further work is needed to locate the active principles from the various extracts and that such

efforts could result in the discovery of new compounds possessing a wide range of bioactivity. The phytochemical analysis of the nine examined *Hypericum* species from the Balkans was published elsewhere (Smelcerovic & Spiteller, 2006).

The results of the total antioxidant capacity assay (Table 3) showed that the methanol extracts of the *Hypericum* species studied possess a significant antioxidant activity. The antioxidant capacity ranged from 540 to 73,100 μmol/g for the TMCEs (expressed as equivalents of α-tocopherol acetate, rounded numbers) and was found to be highest in the case of the TMCE of the flowers of *H. perforatum* (E4), followed closely by *H. barbatum* (aerial parts A2). There seems to be no rule as to the variation of the antioxidant capacity, with the activity also being dependent on the identity of the species and also the site and date of collection.

The antioxidant capacity of the TMCEs of the flower (E2), leaf (E3) and stem (E4) of *H. perforatum* correlated nicely with the known distribution of flavonoids in the plant organs (11.7% in flowers and 7.4% in leaves and stems (Ma & Xiao, 1990)).

The reducing properties are generally associated with the presence of reductons (Pin-Der Duh, 1998). Gordon (1990) reported that the antioxidant action of reductons is based on the breaking of the free radical chain by donation of a hydrogen atom. Reductons also react with certain precursors of peroxide, thus preventing peroxide formation. The data presented above indicate that the marked antioxidant activity seems to be due to the presence of polyphenols that may act in a similar fashion as reductons by donating electrons and reacting with free radicals to convert them to more stable products and, thus, terminate free radical chain reactions. Flavonoids have been shown to have potent antioxidant activity (Ng, Liu, & Wang, 2000), but do not exert the pro-oxidant effects seen with vitamin C and the neurotransmitter dopamine. It is possible, therefore, that the antioxidant actions seen in the present study were due simply to the presence of flavonoids (mentioned above) in the extracts tested, rather than hypericin or hyperforin. The conclusion that the activity is related to the flavonoid derivatives was also made in three recent investigations of the antioxidant activity of *H. perforatum* and *Hypericum triquetrifolium* extracts (Conforti et al., 2002; Silva et al., 2005; Zou et al., 2004). Zou et al. (2004) proposed *H. perforatum* as a dietary supplement or drug for the treatment of various coronary heart diseases, based on its antioxidant activity. The same is true for the species of this study, provided that detailed studies are undertaken to investigate the antioxidant activity *in vivo* and that the active principles are isolated and characterized.

In conclusion, the data from this preliminary screening indicate that these nine *Hypericum* species from the Balkans show excellent antimicrobial and antioxidant activity, which might justify their use in traditional medicine. This also underlines the importance of the ethnobotanical approach for the selection of plants in the discovery of new bioactive substances and places these species among the most promising of indigenous drugs.

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