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Comparative phytochemical and antimicrobial investigations of *Hypericum perforatum* L. subsp. *perforatum* and *H. perforatum* subsp. *angustifolium* (DC.) Gaudin

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The aerial parts of *H. perforatum* subsp. *perforatum* and *H. perforatum* subsp. *angustifolium* were investigated for their chemical composition and antimicrobial activity. Spectrophotometric analysis indicated that *H. perforatum* subsp. *perforatum* is richer in flavonoids and tannins than the other subspecies. HPLC analysis confirmed the higher yield of flavonoids in *H. perforatum* subsp. *perforatum* and gave also a higher content of phenolic acids. *H. perforatum* subsp. *angustifolium* contained more hypericin. The presence of rutin was proven only in *H. perforatum* subsp. *perforatum*. The antimicrobial activity of the extracts of both subspecies was evaluated based on the inhibition zone diameters using the hole-plate diffusion method. The MeOH extracts, dichloromethane and petroleum ether fractions were effective against *Staphylococcus aureus*, *S. epidermidis*, *Enterococcus faecalis* and *Bacillus subtilis*. The results indicate that *H. perforatum* subsp. *angustifolium* had a stronger antimicrobial effect than the other subspecies.

Keywords: *H. perforatum* subsp. *perforatum*, *H. perforatum* subsp. *angustifolium*, flavonoids, phenolic acids, hypericin, tannins, HPLC analysis, antimicrobial activity

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Hypericum perforatum L. (*Hypericaceae* = *Guttiferae*) is a herbaceous, aromatic rhizomatous perennial plant, up to 1 m high, glabrous throughout, with simple, opposite, sessile, ovate to linear leaves sprinkled with oil translucent glands. Flowers are bright yellow and arranged in a broadly paniculate, compound cymose inflorescence at the top of stem. Five petals are asymmetric, with hypericin containing black nodules (1–4). *H. perforatum* is a very variable species and some botanists recognize 3 or even 4 subspecies or varieties based on morphological criteria of differentiation: leaf size, shape and appearance, shape and location of hypericin containing black nodules on petal margins or lami-

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na. Differences in chromosome number are also noted (2, 5–7). Typical subspecies, *H. perforatum* subsp. *perforatum* is primarily a northern and middle European taxon with relatively wide leaves, but towards the south of Europe grow *H. perforatum* subsp. *angustifolium* (DC.) Gaudin, with narrowed lanceolate to linear leaves, and *H. perforatum* subsp. *veronense* (Schrank) Fröhlich, recognized by small ovate to elliptical leaves (3, 8). Only morphological characteristics showing a high degree of variability are insufficient for very exact identification and some other criteria are needed to establish reliable differentiation between subspecies (9–12).

The drug *Hyperici herba* consists of dried flowering tops or aerial parts of typical subspecies. It is used internally in official and folk medicine for the treatment of psychogenic disturbances, depressive states and/or nervous excitements, as an antiphlogistic agent in the treatment of inflammation of the bronchi and urogenital tract, treatment of biliary disorders, bladder irritation, common cold, diabetes mellitus, dyspepsia, as a diuretic, emmenagogue and antimalarial agent, and externally for the treatment and after-treatment of incised and contused wounds, skin ulcers and first-degree burns (13, 14). Chemical investigations of *H. perforatum* subsp. *perforatum* revealed a number of constituents including hypericins, flavonoids, hyperforin as an antibiotic substance, essential oil, tannins and procyanidins (14–18). Antimicrobial investigations of the genus *Hypericum* showed its high activity (19, 20). The extracts of *H. perforatum* subsp. *angustifolium* obtained by high pressure extraction with CO₂ showed antimicrobial activity especially against *Candida albicans* (21).

This study represents the first investigation of the chemical composition and antimicrobial activity of *H. perforatum* subsp. *angustifolium* in comparison to *H. perforatum* subsp. *perforatum*.

EXPERIMENTAL

Plant material

The aerial parts of *H. perforatum* subsp. *angustifolium* were collected in the surroundings of Zadar (Croatia) in July 2002 and determined according to Pignatti (8). The plant material was air-dried at room temperature (20 ± 2 °C). Voucher specimen (collecting No. 353.16) is deposited in the Herbarium Croaticum, Department of Botany, Faculty of Science, University of Zagreb, Croatia.

The aerial parts of *H. perforatum* subsp. *perforatum* were obtained from Apoka Inc. (Austria). The identity and purity were determined by H. Sauer, Institute of Botany, University of Graz, Austria.

Spectrophotometric analysis of flavonoids and tannins

Measurements were carried out using a Specord 50 photometer (Analytik Jena, Germany).

The content of flavonoids, calculated as hyperoside and tannins in plant samples, was determined upon three independent analyses by official Eur. Ph. methods (22). Af-

ter acid hydrolysis (with 25% hydrochloric acid in acetone for 30 minutes at 100 °C), liberated aglycones were spectrometrically determined at 425 nm by forming a complex with AlCl_3 in a methanol/ethyl acetate/acetic acid medium. For the determination of tannin content, water plant extracts were shaken with hide powder for 60 minutes. Hide powder adsorbed tannins. The analysis was carried out spectrometrically at 760 nm after addition of phosphomolybdotungstic reagent in a sodium carbonate medium.

HPLC analysis

Sample preparation. – Powdered plant material (15 g) was extracted with 150 mL methanol at room temperature using a magnetic stirrer for 48 h. The extraction was repeated two times, the combined extracts were filtered and dried in air. The drug-extract-ratios (DER) gave the following values: *H. perforatum* subsp. *angustifolium*, DER = 10.2; *H. perforatum* subsp. *perforatum*, DER = 6.3. The residue was redissolved in 5.0 mL methanol (MeOH extract). The MeOH extracts were shaken with petroleum ether. The upper phases were used for investigations (PE fraction). Subsequently, the MeOH parts were extracted with dichloromethane (DCM fraction).

Standards. – Chlorogenic acid, caffeic acid, rutin, hyperoside, isoquercitrin, quercitrin, quercetin and hypericin were purchased from Roth (Germany). Stock solutions of the standards were prepared as 1 mg mL⁻¹ in HPLC-grade methanol.

All samples were filtered through a 0.45- μm filter (Nalgene 4-mm syringe filters, Nylon) before HPLC analysis (23).

Apparatus and conditions. – The high-performance liquid chromatograph consisted of a LaChrom autosampler L-7200, a pump L-7100, a photodiode array detector L-7450, and an interface D-7000 (all purchased from Merck, Germany), connected to a HSM HPLC system manager and a Hewlett Packard Desk Jet 660c. HPLC grade water and acetonitrile were obtained from Merck (Germany). The compounds were separated on a pre-packed analytical reversed-phase column LiChrospher 100 RP-18 (5 μm , Merck) following a linear gradient using eluents A and B (A = acetonitrile/water/phosphoric acid, 19:80:1, V/V/V; B = acetonitrile/methanol/phosphoric acid, 59:40:1, V/V/V) according to the following profile: 0–8 min 100% A, 8–30 min 100–50% A, 30–45 min 50–0% A, 45–75 min 0% A (15). The flow-rate was kept constant at 1.0 mL min⁻¹ at room temperature (20 \pm 2 °C). Throughout the experiment all injection volumes were 10 μL . The peaks were detected at 254 nm. The identity of HPLC peaks separated by HPLC was confirmed by injection of authentic standards. Variation of the retention time of each peak was less than 1%.

The imprecision of this method on the basis of peak-area ratios for four replicate injections was about 0.5%. Quantification was achieved by the external standard method; the coefficient of correlation for calibration line was 0.9998.

Antimicrobial activity

Preparation of extracts. – The extracts used for antimicrobial testing were prepared according to the procedure described in *Sample preparation*.

Test organisms. – *Staphylococcus aureus* ATCC 25923, *S. epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 1406, *Bacillus subtilis* ATCC 6051, *Escherichia coli* ATCC 25922, *Proteus mirabilis* ATCC 14153 and *Pseudomonas aeruginosa* ATCC 27853 were used as test organisms. The bacteria were incubated on a nutrient agar-slant (stationary culture) for 48 h at 37 °C followed by inoculation in YET-glucose broth. This consisted of 0.5% yeast extract, 1% tryptone (Oxoid L 42, UK), 1% glucose and 1% sodium chloride (pH 7.0). Turbidity was corrected by adding isotonic sodium chloride solution until 10^8 colony-forming units (CFU mL⁻¹) were attained (24).

Hole-plate diffusion method. – Twenty-five mL of sterile Isosensitest agar (Oxoid CM 471, UK) was poured into Petri dishes. The agar was left to set and 0.8 mL of an appropriate bacterial suspension was distributed in it. A 9 mm core of agar was removed from the seeded agar and the hole was closed against the dish bottom with pure agar. Holes were filled up with 0.1 mL of each plant extract. Reference antibacterial substances kanamycin monosulphate (Sigma-Aldrich, Austria) and ampicillin (Merck), were prepared as 1 mg mL⁻¹ water solutions, and diluted with phosphate buffer solution, pH 8 (22). After incubation for 24 h at 37 °C the diameter of the inhibition zones was measured in mm. Three sets of controls were used. One control was the organism control and consisted of a seeded Petri dish with no plant material or standard; in the second control, plant material and standards were introduced in the holes of unseeded Petri dishes to check for sterility; in the third set, the holes of seeded Petri dishes were filled up with solvents (methanol, dichlormethane and petroleum ether) to check their activity (24, 25).

RESULTS AND DISCUSSION

Results of quantitative analyses of flavonoids and tannins showed that *H. perforatum* subsp. *perforatum* ($2.3 \pm 0.0_3\%$ flavonoids, $6.1 \pm 0.0_4\%$ tannins) is richer in these compounds than *H. perforatum* subsp. *angustifolium* ($1.8 \pm 0.0_2\%$ flavonoids, $4.7 \pm 0.0_3\%$ tannins).

Table I. HPLC analysis of phenolic acids, flavonoids and hypericin in methanolic extracts

Compound (%, m/m)	<i>H. perforatum</i> subsp. <i>perforatum</i>	<i>H. perforatum</i> subsp. <i>angustifolium</i>
1 Chlorogenic acid	$0.09 \pm 0.00_1$	$0.04 \pm 0.00_1$
2 Caffeic acid	0.004 ± 0.00	0.001 ± 0.00
3 Rutin	0.70 ± 0.01	–
4 Hyperoside	0.50 ± 0.01	$0.10 \pm 0.00_2$
5 Isoquercitrin	0.40 ± 0.01	$0.05 \pm 0.00_2$
6 Quercitrin	$0.20 \pm 0.00_3$	$0.20 \pm 0.00_4$
7 Quercetin	$0.10 \pm 0.00_1$	$0.01 \pm 0.00_1$
8 Hypericin	$0.03 \pm 0.00_1$	$0.10 \pm 0.00_3$

^a Mean value \pm SD, $n = 4$.

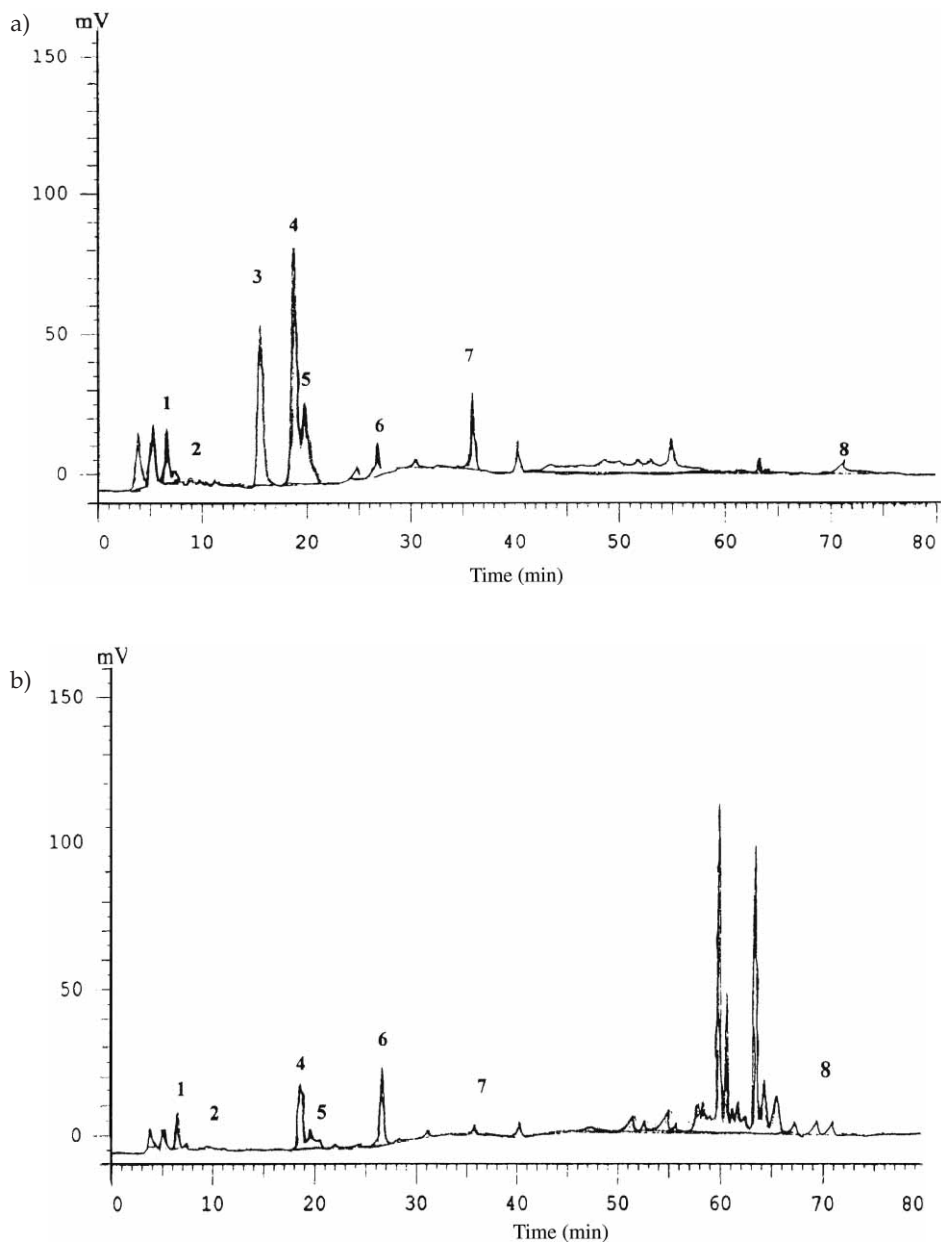


Fig. 1. HPLC chromatograms of a methanolic extract from: a) *Hypericum perforatum* L. subsp. *perforatum*, b) *Hypericum perforatum* L. subsp. *angustifolium* (DC.) Gaudin. Compounds 1–8 are phenolic acids, flavonoids and hypericin – numbering is as given in Table I.

The analysis of methanolic extracts was performed using reversed-phase HPLC. The gradient separation of phenolic acids, flavonoids and hypericin was finished within 80 minutes (Fig. 1). The investigated compounds were identified by comparison of their retention times with those of standards. The identity of HPLC peaks was definitely assessed by co-chromatography after spiking the samples with reference compounds.

The HPLC analysis of the methanolic extracts of investigated subspecies showed similarity in their composition (Table I, Fig. 1). Both subspecies contained chlorogenic acid ($t_R = 6.55$ min), caffeic acid ($t_R = 9.72$ min), hyperoside ($t_R = 18.75$ min), isoquercitrin ($t_R = 19.85$ min), quercitrin ($t_R = 26.74$ min), quercetin ($t_R = 35.91$ min) and hypericin ($t_R = 70.53$ min). The presence of rutin ($t_R = 15.49$ min) was proven only in *H. perforatum* subsp. *perforatum* (Table I, Fig. 1a). Mártonfi *et al.* (17) have also found a chemotype of *H. perforatum* with no rutin.

Quantitative analysis showed higher contents of phenolic acids, hyperoside, isoquercitrin and quercetin in *H. perforatum* subsp. *perforatum*, than in subsp. *angustifolium*, whereas the content of quercitrin was the same in both subspecies (0.20%). *H. perforatum* subsp. *angustifolium* contained a larger quantity of hypericin (0.10%) than the other subspecies (0.03%).

TLC analysis of dichloromethane and petroleum ether fractions of both subspecies confirmed the presence of hypericins and some lipophilic compounds (15).

The results of the hole-plate diffusion method are given in Table II. The methanolic extracts (5 mg mL⁻¹), dichloromethane fractions (5 mg mL⁻¹) and petroleum ether fractions (5 mg mL⁻¹) showed activities against all tested Gram-positive bacteria (*S. aureus*, *S. epidermidis*, *E. faecalis* and *B. subtilis*) by forming clear inhibition zones between 10 and 28 mm. No activity against Gram-negative bacteria (*E. coli*, *P. mirabilis* and *P. aeruginosa*) was observed. The methanolic extracts of *H. perforatum* subsp. *angustifolium* showed higher activity against all tested Gram-positive bacteria than the same extracts of *H.*

Table II. Antibacterial activity of *Hypericum perforatum* subsp. *perforatum* and *H. perforatum* subsp. *angustifolium* extracts

	Inhibition zone diameter (mm) ^a							K	A ^{b,c}
	HP _p (M)	HP _p (DCM)	HP _p (PE)	HP _a (M)	HP _a (DCM)	HP _a (PE)			
<i>Staphylococcus aureus</i>	14 ± 1	18 ± 1	–	15 ± 1	21 ± 1	–	18 ± 0.4	–	
<i>Staphylococcus epidermidis</i>	10 ± 1	–	–	11 ± 1	28 ± 0.4	–	19 ± 1	–	
<i>Enterococcus faecalis</i>	19 ± 1	20 ± 1	15 ± 1	20 ± 1	19 ± 1	12 ± 1	–	17 ± 1	
<i>Bacillus subtilis</i>	–	11 ± 0.3	–	10 ± 1	23 ± 1	–	20 ± 1	–	

^a Mean value ± SD, $n = 3$ (as the diameter of the hole was 9 mm, inhibition zones < 10 mm were not evaluated).

^b Solvent controls (methanol, dichloromethane and petroleum ether) were negative.

^c HP_p – *Hypericum perforatum* subsp. *perforatum*, HP_a – *Hypericum perforatum* subsp. *angustifolium*, M = MeOH extract (500 µg), DCM = dichloromethane fraction (500 µg), PE – petroleum ether fraction (500 µg), K – kanamycin monosulphate (10 µg), A – ampicillin (10 µg).

perforatum subsp. *perforatum*. The largest inhibition zones were noticed against *E. faecalis* for both subspecies (19 and 20 mm). Flavonoids, phenolic acids, hypericins and tannins contribute to the antimicrobial activity of methanolic extracts. The dichloromethane fractions of *H. perforatum* subsp. *angustifolium* produced stronger effects against *S. aureus*, *S. epidermidis* and *B. subtilis* (inhibition zones between 21 and 28 mm) than the same fractions of *H. perforatum* subsp. *perforatum*, but the activity against *E. faecalis* was similar for both subspecies (inhibition zones between 19 and 20 mm). The dichloromethane fractions showed higher activities compared to the methanolic extracts for both subspecies. These activities are assigned to hypericins and lipophilic compounds. The petroleum ether fractions of both plants were found to be active only against *E. faecalis*.

CONCLUSIONS

Chemical investigations indicated that *H. perforatum* subsp. *perforatum* is richer in flavonoids, phenolic acids and tannins than *H. perforatum* subsp. *angustifolium*; the latter contained more hypericin. These biologically active compounds can be considered as antimicrobial agents, because these substances may bind to bacterial adhesins and by doing so they disturb the availability of receptors on the cell surface (24). *H. perforatum* subsp. *angustifolium* showed a stronger antimicrobial activity than the other subspecies. However, as indicated by HPLC chromatograms, full chemical identification of methanolic extracts of both subspecies is needed and is already under way.

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S A Ž E T A K

Usporedna fitokemijska i antimikrobna istraživanja širokolisne i uskolisne rupičaste pljuskavice [*Hypericum perforatum* L. subsp. *perforatum* et *H. perforatum* subsp. *angustifolium* (DC.) Gaudin]

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Istražen je kemijski sastav i antimikrobna aktivnost nadzemnih dijelova širokolisne i uskolisne rupičaste pljuskavice [*H. perforatum* L. subsp. *perforatum* et *H. perforatum* subsp. *angustifolium* (DC.) Gaudin].

Spektrofotometrijskom analizom utvrđena je veća količina flavonoida i trjeslovina u svojiti *H. perforatum* subsp. *perforatum*. Metodom tekućinske kromatografije visoke moći razlučivanja (HPLC) dokazano je da širokolisna rupičasta pljuskavica sadrži veću količinu flavonoida i fenolnih kiselina, dok je veći sadržaj hipericina utvrđen u uskolisnoj rupičastoj pljuskavici. Prisutnost rutina dokazana je samo u svojiti *H. perforatum* subsp. *perforatum*.

Antimikrobna aktivnost ekstrakata istraživanih svojiti ispitana je metodom difuzije. Metanolni ekstrakti, diklormetanske i petroleterске фракције pokazali su učinak na sljedeće mikroorganizme: *Staphylococcus aureus*, *S. epidermidis*, *Enterococcus faecalis* i *Bacillus subtilis*. Svojiti *H. perforatum* subsp. *angustifolium* imala je jači antimikrobni učinak.

Ključne riječi: *H. perforatum* subsp. *perforatum*, *H. perforatum* subsp. *angustifolium*, flavonoidi, fenolne kiseline, hipericin, trjeslovine, HPLC, antimikrobni učinak

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