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Anti-inflammatory and Gastroprotective Properties of *Hypericum richeri* Oil Extracts

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Oil extracts of flowering tops of *Hypericum richeri* Vill. prepared in three different ways were evaluated for chemical composition, and anti-inflammatory and gastroprotective activities. An HPLC method was developed for determination of two dominant flavonoids, quercetin and I3,II8-biapigenin. The carrageenan-induced rat paw edema test was used for screening the anti-inflammatory activity, while indomethacin-induced rat gastric mucosa damage test was used for evaluation of gastroprotective activity. The oil extract prepared by maceration with 96% ethanol, followed by extraction with sunflower oil by heating on a water bath, exhibited the highest anti-inflammatory (38.4 %) and gastroprotective activities (gastric damage score of 0.9). The same oil extract had the highest content of quercetin (49 μg/mL) and I3,II8-biapigenin (60 μg/mL). These results approve the usage of oil extracts *of H. richeri* as an anti-inflammatory and gastroprotective agent.

Keywords: *Hypericum richeri* Vill., oil extracts, quercetin, I3,II8-biapigenin, anti-inflammatory activity, gastroprotective activity.

Hypericum richeri Vill. is one of several species of *Hypericum* L. that has been used in traditional medicine in the central Balkan countries. This plant belongs to the *Drosocarpium* section and grows in mountain regions of south and south-central Europe [1]. Until now, *H. perforatum* L. was the only *Hypericum* species officially accepted in medicine for the treatment of mild to moderate depression, anxiety, and externally for treatment of inflammation of the skin, blunt injuries, wounds and burns [2].

According to our ethnopharmacological investigations, aerial blooming parts of *H. richeri* are used in traditional medicine of Montenegro in the form of an infusion and as an oil extract [3]. The infusion is used for the treatment of different type of inflammation, the common cold, dyspepsia and anxiety, while the oil extract is traditionally used externally for healing skin burns, wounds and injuries, and internally for ulcer treatment. Recent reports of the phytochemical profiling of *H. richeri* revealed the presence of many biological active compounds, such as quercetin and its glycosides, myricetin glycosides, I3,II8-biapigenin, caffeoylquinic acids, hypericin, pseudohypericin, and hyperforin [3-5]. Antimicrobial, antioxidant and anti-inflammatory activity of alcoholic extracts of *H. richeri* have been reported [6,7]. Some less polar compounds, such as quercetin and biapigenin are extractable in oil. It was shown that these flavonoids could contribute to the anti-inflammatory and gastroprotective activity of *H. perforatum* oil extracts (*Oleum Hyperici*) [8].

The present study included chemical characterization of *H. richeri* oil extracts prepared in three different ways, as well as investigation of anti-inflammatory and gastroprotective activity of the extracts in order to justify their ethnomedicinal use.

Table 1: The amount of quercetin and I3,II8-biapigenin in the oil extracts of *Hypericum richeri*.

Sample	Quercetin (μg/mL)	I3,II8-Biapigenin (μg/mL)
Extract 1	27 ± 2	8 ± 1
Extract 2	49 ± 3	60 ± 4
Extract 3	22 ± 2	7 ± 1

Two flavonoids, quercetin and I3,II8-biapigenin, were identified and quantified in the tested oil extracts of *H. richeri*. The amounts of the flavonoids varied between the extracts. Extract 2, prepared by maceration with 96% ethanol, followed by extraction with sunflower oil by heating in a water bath, was characterized by the highest content of both compounds. The flavonoid contents in extracts 1 and 3 are rather similar (Table 1). Ethanol, as a solvent during maceration, probably contributed to a better extraction of dry plant material resulting in a better yield of flavonoids. On the other hand, the condition of the plant material for the extraction (fresh or dry) had less influence on the flavonoid content. In our earlier studies, it was also shown that the procedure for the preparation of *H. perforatum* oil extracts affected the amount of active compounds, but the type of solvent was the most noticeable [8].

The anti-inflammatory activity of the oil extracts administered p.o. in three doses (0.50 mL/kg, 0.75 mL/kg, and 1.25 mL/kg) was studied in comparison with indomethacin by the carrageenan induced paw edema test. Indomethacin, used as a reference drug in a dose of 8 mg/kg, led to a 71% decrease of the edema. All three tested oil extracts exhibited statistically significant anti-inflammatory activity ($P < 0.01$) in a dose dependent manner (Table 2), where only the lowest dose of extract 3 did not show statistically significant activity compared with the control group that received sunflower oil. Extract 2, at a dose of 1.25 mL/kg, exhibited the strongest anti-inflammatory effect (38%). The pure flavonoids, quercetin and I3,II8-biapigenin, administered p.o. at a dose of 8 mg/kg inhibited the edema formation by 59% and 61%, respectively, and was almost in the range of the standard anti-inflammatory drug indomethacin (Table 2). The anti-inflammatory effect of quercetin administrated locally on the acute inflammatory process has been previously demonstrated [9,10]. Taking into account that the anti-inflammatory effect of these flavonoids has been shown, and that the highest content of both compounds was noticed in extract 2, it is expected that this extract would possess the strongest anti-inflammatory activity. In our previous paper, oil extract of *H. perforatum* obtained by maceration with ethanol also possessed the highest anti-inflammatory effect. Comparing these two *Hypericum* species, *H. perforatum* oil extracts were more active, probably due to a greater amount of quercetin [8].

Table 2: Effects of the oil extracts of *Hypericum richeri* on carrageenan induced rat paw edema and on gastric ulcer after p.o. administration

Groups	Dose (mL/kg)	Anti-inflammatory effect (%)	Gastric damage score
Control [a]	/	/	3.5 ± 1.2
Extract 1	0.5	14.9 ± 4.2**	2.7 ± 1.2
	0.75	16.8 ± 7.7**	2.3 ± 1.0*
	1.25	24.7 ± 4.2**	1.8 ± 0.5**
Extract 2	0.5	30.7 ± 9.5**	1.4 ± 0.4*
	0.75	35.3 ± 5.8**	1.2 ± 0.5**
	1.25	38.4 ± 7.8**	0.9 ± 0.5**
Extract 3	0.5	10.7 ± 3.5	2.8 ± 0.7
	0.75	21.4 ± 6.4**	2.7 ± 0.8*
	1.25	26.3 ± 7.0**	2.0 ± 1.0*
Indomethacin [b]	8	71.5 ± 15.9**	3.8 ± 0.9
Quercetin [b]	8	59.0 ±14.7**	0.3 ± 0.2**
I3,II8-Biapigenin	8	60.9 ± 20.6**	0.2 ± 0.2**
Ranitidine [b]	20	/	0.5 ± 0.3**

[a] Sunflower oil, p.o.
[b] dose of standard is expressed in mg/kg
Data are presented as the mean ± S.D. (n=6).
* $P < 0.05$ significantly different from control group
** $P < 0.01$ significantly different from control group (Mann-Whitney *U* test)

Indomethacin, as one of the non-steroid anti-inflammatory drugs that inhibits prostaglandin production, can lead to the formation of gastrointestinal ulcer. However, administration of the *H. richeri* oil extracts given immediately after indomethacin, significantly ($P < 0.01$) reduced the gastric lesions, except for the lowest doses of extracts 1 and 3. Extract 2, at a dose of 1.25 mL/kg, showed the highest activity, with the lowest gastric damage score of 0.9 (Table 2). Pure quercetin and I3,II8-biapigenin also significantly reduced the gastric lesions, with a gastroprotective activity similar to that of ranitidine. Such results are in accordance with our previous investigations of oil extracts of *H. perforatum* [8].

The results obtained in this study provide evidence for the ethnopharmacological usage of oil extracts *of H. richeri* as an anti-inflammatory and gastroprotective agent. Quercetin and I3,II8-biapigenin, as identified flavonoids in the tested oil extracts, could be partially responsible for these activities. In both experimental models, extract 2 showed the highest activity, probably due to the greatest amount of quercetin and I3,II8-biapigenin caused by pre-extraction of plant material with ethanol. However, comparison of the results obtained for the anti-inflammatory and gastroprotective activity of *H. perforatum* oil extracts (*Oleum Hyperici*) [8] and oil extracts of *H. richeri* indicated that it is more favorable to use *H. perforatum* than *H. richeri*.

Experimental

Plant material: The flowering tops of *H. richeri* Vill. were collected in July 2005 on Bogićevica mountain, Montenegro. The voucher specimen No. 314/05 has been deposited at the Biology Department, Faculty of Science, University of Montenegro, Podgorica.

Preparation of the oil extract: Oil extracts were prepared in 3 different ways. The first (extract 1) was prepared by maceration of 60 g of fresh flowering tops in 300 g of sunflower oil exposed to sunlight for 40 days; 290 g of extract was obtained. The second oil extract (extract 2) was prepared by maceration of 20 g of dried plant material with 60 mL 96% ethanol for 24 h at room temperature. After that, extraction was continued by the addition of 120 g sunflower oil, followed by evaporation of the ethanol by heating the mixture for 4 h in a warm bath yielding 110 g of oil extract. The third oil extract (extract 3) was prepared by digestion of 20 g dried flowering tops in 200 g sunflower oil for 3 h yielding 190 g of the oil extract. The obtained extracts were filtered and kept at room temperature.

HPLC analysis: For HPLC analysis of the extracts, further re-extraction with methanol was done. A 50 mL portion of each oil extract was re-extracted 3 times with 50 mL methanol. The methanol extracts were combined, evaporated under vacuum at a temperature below 50°C and concentrated to 10 mL. The samples were filtered through a 0.45 μm filter prior to injection. Analyses were carried out on a Hewlett Packard HPLC model 1090; DAD detector (HP 1040); column Lichrospher RP-18 (5 μm, 250 × 4 mm i.d.) (Merck); flow rate 1 mL/min; mobile phase: A (99% H_2O, 1% H_3PO_4), B (acetonitrile); elution by combination of gradient and isocratic modes: 16–36% B, 0–6 min; 36–60% B, 6–12 min, 60-80% B, 12–16 min, 80–100% B, 16–20 min, 100% B, 20–35 min. The absorption was measured at 270, 350 and 590 nm. The injection volume was 20 μL. Quercetin and I3,II8-biapigenin were identified by the co-injection method, using the standard compounds previously isolated in our laboratory [3]. The amounts of these compounds were calculated using calibration curves. Detection was performed at 270 nm. Concentrations used for calibration were 0.03-0.2 mg/mL for I3,II8-biapigenin and 0.03-0.5 mg/mL for quercetin. All experiments were conducted in triplicate. The results are presented as μg/mL of the oil extract.

Animals: Male, 8-week-old Wistar rats (200-250 g) were purchased from the Military Medical Academy Animal House, Belgrade. They were housed in a local animal house with a controlled light cycle and were given commercial food pellets and water *ad libitum*. Each experimental group consisted of 6 animals. The animals were deprived of food for 18-20 h before the beginning of experiments with free access to tap water. Throughout the studies the protocol for these experiments was approved by the Institutional Animal Care and Use Committee.

Anti-inflammatory activity: The carrageenan-induced rat paw edema test has been used as an experimental model for screening the anti-inflammatory activity of the oil extracts and pure compounds according to the modified method of Oyanagui and Sato [11]. The extracts were administered p.o. in different doses (0.50 mL/kg, 0.75 mL/kg, and 1.25 mL/kg). Indomethacin, dissolved in DMSO, was used as a reference drug at a dose of 8 mg/kg p.o. Quercetin and I3,II8-biapigenin, dissolved in sunflower oil, were used at a dose of 8 mg/kg p.o. The control animals were given sunflower oil in a dose of 1.25 mL/kg p.o. Carrageenan-saline solution (0.5% in a volume of 0.1 mL) was injected into the plantar surface of the right hind paw 1 h after the oral administration of the sunflower oil, oil extracts, quercetin, I3,II8-biapigenin or indomethacin. A pure saline solution (0.9%, 0.1 mL) was injected into the left hind paw, which served as the control (non-inflamed paw). The animals were sacrificed by ether anesthesia 3 h after the carrageenan injection and the paws were cut off for weighing. A difference in weight between the right and left paw, treated versus untreated (control) rats, served as an indicator of the inflammatory response intensity (i.e. anti-inflammatory activity). It was calculated from the expression:

Inflammatory response intensity (%)=$(\Delta k - \Delta e)\times 100/\Delta k$

where Δk is the difference in the paw weight in the control group and Δe is the difference in the paw weight in the treatment groups.

Gastroprotective activity: In order to study the gastroprotective activity of the oil extracts and pure compounds, an experimental model of acute gastric mucosal damage induced by indomethacin in a dose of 8 mg/kg p.o. was utilized. Extracts and sunflower oil were given in different doses (0.50 mL/kg, 0.75 mL/kg, and 1.25 mL/kg) p.o. immediately after indomethacin. Quercetin and I3,II8-biapigenin, dissolved in sunflower oil, were used in a dose of 8 mg/kg p.o. Ranitidine (20 mg/kg p.o.) was used as a reference drug. The animals were sacrificed 4 h after that and their stomachs were removed and opened along the greater curvature. Lesions were examined under an illuminated magnifier (3×). The intensity of gastric lesions was assessed according to the total length of gastric lesions (mm) and the lesion area (mm^2), as well as to a modified scoring system of Adami *et al*. [12].

Statistical analysis: Data are presented as mean ± standard deviation (SD). The Mann-Whitney *U*-test was applied using the program SPSS11.5 for Windows for comparison of data between groups. Differences were considered statistically significant at $P < 0.05$.

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