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Original article

Secondary Metabolites of *Hypericum* L. Species as Xanthine Oxidase Inhibitors

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SUMMARY

Nine *Hypericum* species (*H. barbatum, H. hirsutum, H. linarioides, H. olympicum, H. perforatum, H. rochelii, H. rumeliacum, H. tetrapterum* and *H. umbellatum*) collected in Serbia were assayed for inhibitory potential against xanthine oxidase *in vitro*, on the commercial enzyme, and compared with allopurinol. Seven studied *Hypericum* species (*H. barbatum, H. rochelii, H. rumeliacum, H. umbellatum, H. perforatum, H. tetrapterum* and *H. olympicum*) inhibit commercial xanthine oxidase with an IC_{50} below 100 μg/mL. *H. barbatum* exerted the most potent inhibitory effect (IC_{50} = 31.84 ± 6.64 μg/mL), followed closely by *H. perforatum* (IC_{50} = 37.12 ± 4.06 μg/mL).

Key words: **xanthine oxidase inhibition, *Hypericum*, secondary metabolites**

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INTRODUCTION

Xanthine oxidase (XO) is a validated target for therapeutic treatment of gout, hyperuricemia and associated conditions, with a few XO inhibitors present on the market (1). Levels of XO in plasma are enhanced also in ischemia-reperfusion injury, hemorrhagic shock, cholecystis, hypercholesterolemia, adult respiratory distress syndrome, carcinogenesis, which provides additional indications where XO inhibitors may exert their therapeutic potential (2).

Secondary metabolites from plants have a long tradition of being used as therapeutics in medicine (3). *Hypericum* species are traditionally used as medicinal plants all over the world (4). *H. perforatum* (St. John's wort) is one of the best chemically determined species. The investigation of *Hypericum* species have increased over the years due to evidenced bioactivities of compounds found in *H. perforatum*. Chemically, naphthodianthrones, primarily hypericin and pseudo-hypericin, phloroglucinol derivatives, especially hyperforin, and flavonoids quercetin, quercitrin, hyperoside and rutin represent the main constituents in the *Hypericum* species (5).

Continuing our research on the chemical composition (6-11) and pharmacological activities (11-14) of *Hypericum* species, in the present study extracts of nine *Hypericum* species (*H. barbatum*, *H. hirsutum*, *H. linarioides*, *H. olympicum*, *H. perforatum*, *H. rochelii*, *H. rumeliacum*, *H. tetrapterum* and *H. umbellatum*), collected in Serbia, were evaluated for inhibitory potential against XO *in vitro*, on the commercial enzyme.

MATERIALS AND METHODS

Plant material

Table 1 contains data on the identity of nine assayed *Hypericum* species, their taxonomic placement within sections of the genus *Hypericum*, voucher numbers of the deposited herbarium specimens (Herbarium Moesicum Doljevac, Serbia), collection period and locality (15). Collection was done in the blooming stage.

Table 1. **Data on the assayed *Hypericum* species**

Section	Plant species	Voucher number (HMD)	Collection period	Locality
Drosocarpium Spach	*H. barbatum*	7280	August 2010	Suva planina, East Serbia
	H. rochelii	6578	July 2010	Sokobanja, East Serbia
	H. rumeliacum	7287	July 2010	Suva planina, East Serbia
	H. umbellatum	6579	July 2010	Surroundings of Pirot, South East Serbia
Hypericum	*H. perforatum*	7291	July 2010	Surroundings of Leskovac, South Serbia
	H. tetrapterum	8223	July 2010	Bosilegrad, South East Serbia
Olympia Spach	*H. olympicum*	7288	July 2010	Rujan planina, South Serbia
Taeniocarpium Jaub. & Spach	*H. hirsutum*	7282	July 2010	Suva planina, East Serbia
	H. linarioides	8224	July 2010	Suva planina, East Serbia

Preparation of plant extracts

The extractions were performed using 3 g dry plant material and 30 mL of ethanol, using an indirect sonication method. Sonications lasted 30 min, using a Bandelin Sonorex Digital 10 P apparatus (Bandelin). At the end of extraction, filtration was done in order to separate the extracts from the residual plant material. After washing the residues with 15 mL of ethanol, the filtrates were combined and evaporated using a rotary vacuum evaporator Büchi® Rotavapor® R-210 (Büchi). Dry extracts were then dissolved in dimethyl sulfoxide (DMSO) and stored at -20 ° C until measurement.

Evaluation of xanthine oxidase inhibition

Inhibition of XO was evaluated *in vitro* on the commercial bovine milk enzyme (Sigma-Aldrich), by spectrophotometric measurement of uric acid formation at 293 nm. The assay was performed in a series of test-tubes (total volume 2150 μL) prepared in the following order: i) Test samples: 0.01 units of XO, one of the studied extracts diluted in DMSO (purity ≥ 99.5%; Sigma-Aldrich) (the final concentration of DMSO in the assay was 4.65 % v/v), 232.5 μM of xanthine (purity ≥ 99.5%; Sigma-Aldrich), and 46.5 mM TRIS-HCl buffer (pH 7.8); ii) Solvent control samples: the same amount of XO, appropriate amount of DMSO, xanthine and TRIS-HCl buffer; iii) Control samples: the same amount of XO, xanthine and TRIS-HCl buffer adjusted to the same volume. Blank samples were prepared for each group (i-iii). The tubes were incubated at 37 °C for 15 min, and after that the reaction was stopped by adding 100 μL of perchloric acid. The difference in absorbance, calculated as a percent change of the control with appropriate amount of DMSO, which correlates to uric acid formation, was used for the determination of the percentage of enzyme inhibition. DMSO, at a final concentration of 4.65 % v/v, did not affect the enzyme assay. The evaluation of XO inhibitory potential of all samples was performed at the concentrations of 100 μg/mL. Extracts exerting inhibition greater than 50 % at 100 μg/mL were tested in a broader concentration range in order to allow calculation of IC_{50} values. Each IC_{50} curve was generated using four concentrations of inhibitor producing an inhibition. Positive control was allopurinol and experiments were performed in triplicate.

RESULTS AND DISCUSSION

Seven of the nine studied *Hypericum* species inhibit commercial bovine milk XO with an IC_{50} below 100 μg/mL (Table 2). *H. barbatum* showed the most potent inhibitory effect (IC_{50} = 31.84 ± 6.64 μg/mL). Allopurinol, an approved XO inhibitor, exhibited stronger inhibitory potential (IC_{50} = 0.20 ± 0.03 μg/mL) than studied *Hypericum* species. The extracts of *Hypericum* species belonging to the section *Taeniocarpium* did not inhibit commercial bovine milk XO with an IC_{50} below 100 μg/mL.

Table 2. **Inhibitory activities of studied *Hypericum* species against bovine milk xanthine oxidase (IC_{50}, μg/mL)**

Allopurinol		0.20 ± 0.03
Section	**Plant species**	**IC_{50} (μg/mL) XO**
Drosocarpium Spach	*H. barbatum*	31.84 ± 6.64
	H. rochelii	42.78 ± 9.32
	H. rumeliacum	43.70 ± 5.85
	H. umbellatum	41.40 ± 7.14
Hypericum	*H. perforatum*	37.12 ± 4.06
	H. tetrapterum	48.77 ± 7.21
Olympia Spach	*H. olympicum*	64.76 ± 9.14
Taeniocarpium Jaub. & Spach	*H. hirsutum*	> 100
	H. linarioides	> 100

Benedí et al. (16) determined IC_{50} 68.30 μg/mL for the hydroethanolic standardized extract of *H. perforatum* (0.3% total hypericins), while Havlik et al. (17) determined IC_{50} 46.70 μg/mL for the 80% aqueous ethanolic extract of *H. perforatum* and IC_{50} 55.40 μg/mL for the methylene chloride - methanolic (50/50 CH_2Cl_2/MeOH) extract of *H. perforatum*.

We found earlier that there is a strong correlation between secondary metabolite contents and the infrageneric classification of Robson (15) among the nine *Hypericum* species (*H. barbatum*, *H. hirsutum*, *H. linarioides*, *H. maculatum*, *H. olympicum*, *H. perforatum*, *H. richeri*, *H. rumeliacum* and *H. tetrapterum*), collected on different locations in Serbia and the F.Y.R. Macedonia (6). It was shown that *H. barbatum* possesses higher (3.9 times) content of hypericin than *H. perforatum* (6), which is in agreement with the study of Kitanov (18), who reported that the content of total hypericins in *H. barbatum* is by 2.4 times higher than in *H. perforatum*. The potent inhibitory effect of *H. barbatum* against XO activity may be related to the high level of hypericin. Also, *H. perforatum* showed a very potent inhibitory effect against XO with an IC_{50} value of 37.12 ± 4.06 μg/mL. The highest content of hyperforin was found in *H. perforatum* (6).

CONCLUSION

Ethanolic extracts of *H. barbatum*, *H. rochelii*, *H. rumeliacum*, *H. umbellatum*, *H. perforatum*, *H. tetrapterum* and *H. olympicum* inhibit commercial bovine milk XO with an IC_{50} below 100 μg/mL. *H. barbatum* showed the highest inhibitory activity and may be potentially used in the treatment of hyperuricemia and gout.

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Inhibicija ksantin-oksidaze sekundarnim metabolitima iz biljnih vrsta roda *Hypericum* L.

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SAŽETAK

Ispitan je uticaj devet *Hypericum* vrsta (*H. barbatum, H. hirsutum, H. linarioides, H. olympicum, H. perforatum, H. rochelii, H. rumeliacum, H. tetrapterum* i *H. umbellatum*) prikupljenih na području Srbije na aktivnost komercijalne ksantin-oksidaze *in vitro* i upoređena sa alopurinolom. Sedam ispitivanih *Hypericum* vrsta (*H. barbatum, H. rochelii, H. rumeliacum, H. umbellatum, H. perforatum, H. tetrapterum* i *H. olympicum*) inhibiraju komercijalnu ksantin-oksidazu sa IC_{50} vrednostima nižim od 100 μg/mL. *H. barbatum* (IC50 = 31,84 ± 6,64 μg/mL) i *H. perforatum* (IC50 = 37,12 ± 4,06 μg/mL) su se pokazali kao najefikasniji inhibitori ksantin-oksidaze.

Ključne reči: **inhibicija ksantin-oksidaze, *Hypericum*, sekundarni metaboliti**