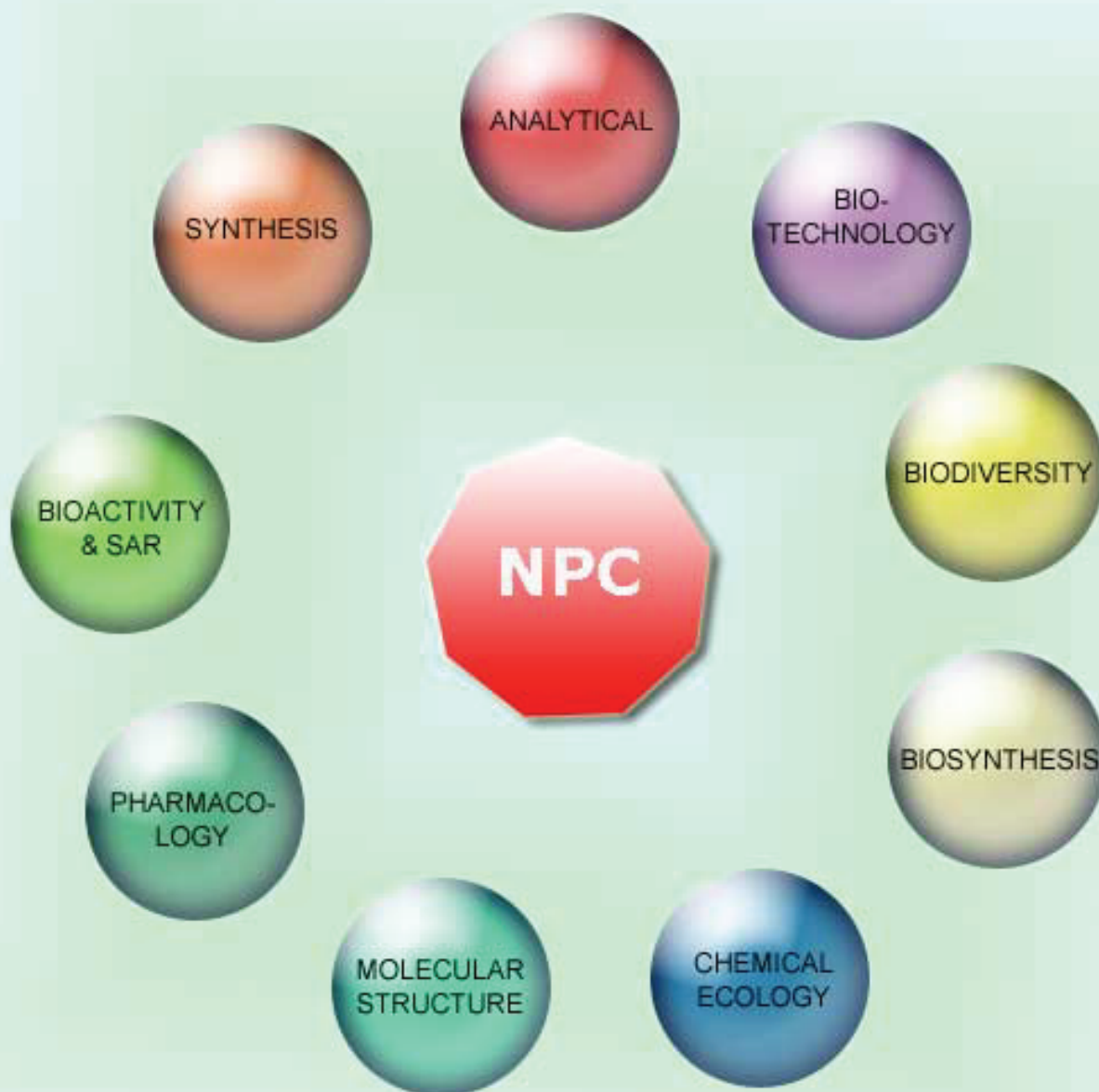


# NATURAL PRODUCT COMMUNICATIONS

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## Anti-inflammatory Activity of *Melampyrum barbatum* and Isolation of Iridoid and Flavonoid Compounds

Erzsébet Háznagy-Radnai<sup>a</sup>, Laura Fási<sup>a</sup>, Edit Wéber<sup>b</sup>, Gyula Pinke<sup>c</sup>, Gergely Király<sup>d</sup>, Anita Sztojkov-Ivanov<sup>e</sup>, Róbert Gáspár<sup>e</sup> and Judit Hohmann<sup>a,f\*</sup>

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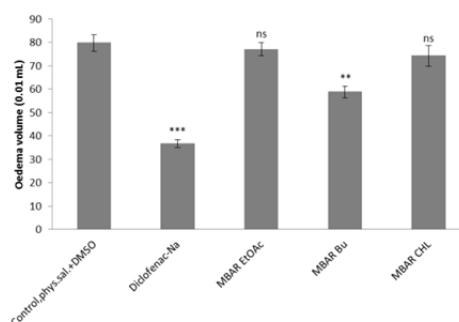
*Melampyrum barbatum* Waldst. & Kit. ex Willd. (Scrophulariaceae) has been used in traditional medicine for the treatment of rheumatic complaints and different skin diseases. In the course of our study the anti-inflammatory activity of the aerial parts of *M. barbatum* was evaluated. A MeOH extract was prepared and consecutively partitioned with CHCl<sub>3</sub>, EtOAc and *n*-BuOH. The fractions were assayed in *in vivo* carrageenan-induced rat paw oedema model. The intraperitoneally administered *n*-BuOH phase exerted marked inhibitory effect (33.6 %,  $p < 0.01$ ). Multistep chromatographic separation afforded musaenoside and aucubine from *n*-BuOH fraction. Moreover, 8-epiloganin, loganic acid and musaenoside were obtained from EtOAc fraction and apigenin, luteolin, benzoic acid and galactitol from CHCl<sub>3</sub> fraction. These data validate the ethnomedicinal use of *M. barbatum* for the treatment of inflammatory diseases and reveal that iridoids and flavonoids could be responsible for the anti-inflammatory effect of this species.

**Keywords:** *Melampyrum barbatum*, Scrophulariaceae, Iridoids, Flavonoids, Anti-inflammatory activity.

*Melampyrum* species (Scrophulariaceae) were widely applied in traditional medicine as anticonvulsant, sedative, cardiovascular and anti-inflammatory agents [1,2]. Chemical and pharmacological studies of *Melampyrum* genus afforded the identification of flavonoids, phenylcarboxylic acids, alkaloids, iridoids, triterpenoids and sterols [3-6]. Pharmacological studies demonstrated that *Melampyrum* species have antioxidant, free radical scavenging and anti-inflammatory properties, and sedative effect targeting GABAergic neurotransmission [2,7,8]. No data on the chemical constituents of this species have been reported earlier.

In the present work the aerial parts of *M. barbatum*, collected from wild stock in Hungary, were extracted with MeOH-H<sub>2</sub>O 7:3, and then liquid-liquid partition was performed with CHCl<sub>3</sub>, EtOAc and *n*-BuOH. These fractions were examined for anti-inflammatory activity using *in vivo* carrageenan-induced rat paw oedema test after intraperitoneal (i.p.) administration of the samples. The *n*-BuOH extract (MBAR Bu) had pronounced inhibitory effect (33.6 %,  $p < 0.01$ ) at 10 mg/kg dose, whereas the EtOAc and CHCl<sub>3</sub> extracts (MBAR EtOAc and MBAR CHL) did not influence the intensity of the inflammatory reaction significantly ( $p > 0.05$ ) (Figure 1).

The CHCl<sub>3</sub>, EtOAc and *n*-BuOH fractions of *M. barbatum* were subjected CC, VLC and preparative TLC separations. The isolated compounds were identified by 1D and 2D NMR analyses. Apigenin, luteolin, benzoic acid and galactitol were isolated from CHCl<sub>3</sub> fraction; 8-epiloganin, loganic acid and musaenoside from EtOAc fraction; and musaenoside and aucubin were obtained from the *n*-BuOH fraction.



**Figure 1:** Inhibitory effects of *M. barbatum* extracts (10 mg/kg, i.p.) and diclofenac-Na (5 mg/kg, i.p.) on carrageenan-induced paw oedema volume in rats. Each column indicates the mean  $\pm$  SEM. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; compared with physiological saline and DMSO (5 mL/kg) in the control group.

Recent studies have shown the anti-inflammatory activity of aucubin in the same *in vivo* model (inhibition 37.9%,  $p < 0.001$  at 5.0 mg/kg) [9]. The anti-inflammatory effect of 8-epiloganin and musaenoside was investigated, and found that both compounds suppressed the production of nitric oxide (NO) and prostaglandin E<sub>2</sub>, and the expression of inducible NO synthase and cyclooxygenase-2 (COX-2) induced by lipopolysaccharide (LPS) in the RAW264.7 murine macrophage cell line. 8-Epiloganin and musaenoside also inhibited the release of pro-inflammatory cytokines induced by LPS, namely, tumor necrosis factor- $\alpha$  and interleukin-1 $\beta$  [10]. Anti-inflammatory effects of loganic acid on carrageenan-induced mouse paw oedema and TPA-induced mouse ear oedema models was demonstrated [11]. Moreover, loganic acid

was found to be a potent anti-inflammatory agent when iridoids were evaluated for their potential to inhibit COX-1 and COX-2 enzymes. In these assays, loganic acid exhibited COX-1 (36.0±0.6%) and COX-2 (80.8±4.0%) inhibition at 10 µM concentration [12]. Apigenin and luteolin were found to significantly inhibit TNF $\alpha$ -induced NF- $\kappa$ B transcriptional activation due to inhibition of the activity of GAL4-NF- $\kappa$ B p65 fusion protein. Furthermore, the administration of apigenin and luteolin markedly inhibited acute carrageenan-induced paw edema in mice [13].

All of the above studies indicate that iridoids and flavonoids are responsible for the anti-inflammatory activity of *M. barbatum* extract. The highest activity of the *n*-BuOH phase most probably is the consequence of the high accumulation of iridoids in this fraction. Our study validates the ethnomedicinal use of the plant for the treatment of inflammatory diseases, and are in good agreement with earlier results reported for *M. pratense*, which anti-inflammatory activity was proved on peroxisome proliferator-activated receptors- (PPARs)- $\alpha$  and - $\gamma$ , activation of NF- $\kappa$ B, induction of interleukin-8 (IL-8) and E-selectin *in vitro* [2].

### Experimental

**Plant material:** *M. barbatum* Waldst. & Kit. ex Willd. was gathered in Öskü (Hungary) in July 2013 and identified by Gy. Pinke and G. Király. A voucher specimen (MBAR No 35) was deposited in the Herbarium of Institute of Pharmacognosy, University of Szeged. The plant material was stored at -20 °C until processing.

**Extraction and Isolation:** The ground aerial parts (4 kg) were extracted with 17 L MeOH-H<sub>2</sub>O 7:3 for 3×20 minutes using an ultrasonic bath. The extract obtained was concentrated in vacuum and fractionated using solvent-solvent partition with CHCl<sub>3</sub> (3.5 L), EtOAc (3.5 L) and *n*-BuOH (3.5 L). On evaporation of the CHCl<sub>3</sub> phase, galactitol was crystallised. The mother liquor of CHCl<sub>3</sub> phase (10.88 g) was subjected to CC on polyamide using MeOH-H<sub>2</sub>O mixtures (1:4, 2:3, 3:2, 4:1, v/v) as eluents. In MeOH-H<sub>2</sub>O (2:3) eluate crystal formation (benzoic acid) was observed. Fraction obtained with MeOH-H<sub>2</sub>O (3:2) was separated

by prep TLC on silica gel with *n*-hexane-acetone (3:2, v/v), yielding apigenin (5.9 mg) and luteolin (10.2 mg). The EtOAc fraction (2.98 g) was purified by prep TLC on silica gel using CHCl<sub>3</sub>-MeOH (4:1, v/v) as eluent. The detection was performed by spraying with *p*-dimethylamino-benzaldehyde + cc. HCl solution. By this means mussaenoside (56.4 mg) [5], 8-epiloganin (15.6 mg) [5] and loganic acid (12.2 mg) [3] were isolated. The *n*-BuOH phase (33.48 g) was separated by VLC on silica gel G (15 µm, Merck) using gradient system of CH<sub>2</sub>Cl<sub>2</sub>-EtOAc-MeOH-H<sub>2</sub>O (1:1:0:0, EtOAc, 0:4:1:0, 0:1:1:0, MeOH, 0:0:1:1 v/v/v/v), to obtain 13 fractions (I-XIII). Fraction VI was further purified by preparative TLC on silica gel with EtOAc-MeOH-H<sub>2</sub>O (5:2:3, v/v/v), affording mussaenoside (18.6 mg) and aucubin (49.6 mg) [5].

**In vivo anti-inflammatory effect on carrageenan-induced paw oedema model:** The anti-inflammatory effect was investigated in carrageenan-induced inflammatory paw oedema model in rats as described in ref [9]. The animals were treated in accordance with 86/609/ECC Directives and the Hungarian Act for the Protection of Animals in Research (Article 32 of Act XXVIII), with the approval of the Hungarian Ethics Committee for Animal Research (No IV/198/2013). Each experimental group comprised ten rats. The animals received 10 mg/kg i.p. of the CHCl<sub>3</sub>, EtOAc and *n*-BuOH phases. Physiological saline-DMSO mixture was used as control. Diclofenac-Na (5 mg/kg i.p.) was administered as positive control (inhibition 37%, *p* < 0.001).

Statistical analysis was performed with Prism 5.0 software (GraphPad, San Diego, CA, USA). The differences in the extents of paw oedema between the treated and control groups were determined by one-way analysis of variance (ANOVA) with Dunett's test. The criterion for statistical significance was *p* < 0.05. All values are expressed as mean ± SEM.

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