SUPPLEMENTARY MATERIAL

***In vitro* anti-denaturation and anti-hyaluronidase activities of extracts and galactolipids from leaves of *Impatiens parviflora* DC.**

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Abstract

The *in vitro* anti-denaturation and anti-hyaluronidase activity of *Impatiens parviflora* extracts and isolated galactolipids (MGDG-1, DGDG-1) were investigated. This is the first report on these compounds in *I. parviflora*. All extracts showed anti-hyaluronidase activity but only methanolic extract from fresh leaves exhibited significant activity against heat induced denaturation of BSA in a dose dependent manner. At 500 µg/mL the extract and the reference drug showed 79.05% and 99.81% inhibition of protein denaturation, respectively. These results indicate that fresh leaves of *I. parviflora* may be beneficial in inflammatory conditions, especially those associated with protein denaturation, such as rheumatoid arthritis. The study revealed that only MGDG-1 showed weak activity in anti-denaturation assay but both galactolipids were potent inhibitors of hyaluronidase. MGDG-1 completely inhibited the enzyme activity at concentration 127.9 µg/mL. These results indicate the potential of galactolipids in the treatment of diseases associated with the loss of hyaluronic acid.

Keywords: *Impatiens parviflora*, anti-denaturation, anti-hyaluronidase, anti-arthritic, (MGDG) monogalactosyldiacylglycerol, (DGDG) digalactosyldiacylglycerol,

Experimental

Standards and Reagents. Methanol, chloroform, acetic acid were obtained from POCH (Gliwice, Poland). DMSO, Albumin from bovine serum: fraction V $\geq 98\%$ (A3294), Hyaluronidase from bovine testes type I-S, *Streptococcus equi* hyaluronic acid, diclofenac sodium, D-glucose, L-glucose, D-arabinose, and L-arabinose were obtained from Sigma-Aldrich. Galactolipid standards (monogalactosyldiacylglycerol –MGDG, digalactosyldiacylglycerol –DGDG) were purchased from Lipid Products (Nutfield Nurseries, UK). All reagents used were of analytical grade.

Plant Material. The leaves of *Impatiens parviflora* DC. were collected in July 2012 near Cracow, Poland. The plant material was identified by Dr Agnieszka Szewczyk (Department of Pharmaceutical Botany, Jagiellonian University, Cracow, Poland). A voucher sample (Reference no.: FG 810/2012) of dried leaves has been deposited in the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Medical College, Jagiellonian University, Cracow. A sample of fresh plant material (Reference no.: FG 811/2012) has been stored in -20°C .

Extraction. Dried and powdered plant material (20 g) was extracted with CHCl_3 (2 times, 2 x 50 ml, for 2h) and then with MeOH (2 times, 2 x 50 ml, for 2h) on a boiling water bath under reflux. The combined CHCl_3 and MeOH extracts were concentrated *in vacuo* to yield 1.8 g and 4.1 g of viscous brown residues, respectively. Fresh leaves (20 g) were processed immediately after collection of plant material by immersion in boiling MeOH to deactivate enzymes and then were extracted by maceration with MeOH (100 ml) at room temperature for 24h. The obtained crude stabilized extract was evaporated in a rotary evaporator under reduced pressure to obtain green residue (0.8 g).

Preliminary Phytochemical Analysis. Portions of extracts were subjected to phytochemical analysis for the presence of phenols, flavonoids, coumarins, triterpenes, saponins, carotenoids, sterols, and lipids, by using standard methods (Harborne 1984; Wagner & Bladt 1996).

Isolation. In order to isolate galactolipids (MGDG and DGDG), extracts from a larger sample of fresh leaves (100 g) were prepared in an analogous manner as described above.

The MeOH extract (4.2 g) was then fractionated by means of medium-pressure chromatography (MPLC) using the following isocratic solvent system: CHCl_3 – CH_3OH – CH_3COOH (40:10:1 v/v). In this work a Büchi Sepacore MPLC apparatus equipped with two pump modules C-601 and C-605 connected with pump manager C-610 and fraction collector C-660 was used. The MPLC conditions were as follows: silica gel 60 (Lichroprep Si 60, Merck), column (40 mm x 150 mm), flow rate 13 mL/min. The collected fractions were examined by TLC (silica gel 60 plates Merck) in the solvent system CHCl_3 – CH_3OH – CH_3COOH (40:10:1 v/v). Chromatograms were visualized by spraying the TLC plates with 25% H_2SO_4

in MeOH, followed by heating (120°C). MGDG and DGDG were identified by comparison of *R_f* values with MGDG and DGDG standards (Lipid Products, Nutfield Nurseries, UK).

Thus, 6 pooled fractions were obtained (Fr I-VI). The fraction rich in MGDGs (FrII) was further purified by means of preparative TLC (Analtech, Silica Gel G, 20 x 20 cm, 500 microns) in the solvent system CHCl₃-CH₃OH-CH₃COOH (40:10:1 v/v). Galactolipids were eluted with CHCl₃-CH₃OH mixture (2:1 v/v) to yield 30 mg of MGDG-1. In the same manner purification of fraction FrIV gave 28 mg of DGDG-1.

Hydrolysis. Acid hydrolysis of isolated MGDG-1 and DGDG-1 was performed on TLC plates using HCl *in statu nascendi* for 30 min at 60°C. The plates were developed twice in CHCl₃-CH₃OH-H₂O (23:12:2 v/v) together with sugar standards. Sugars were identified following spraying the TLC plate with aniline phthalate and heating (120°C, 20 min) (Janeczko et al.1990).

Fatty Acid Analysis. MGDG-1 and DGDG-1 were converted to methyl esters according to AOAC Official Method 991.39 and then subjected to GLC analysis (AOAC 1995). GLC analysis was carried out on TRACE GC Ultra Chromatograph, (Thermo Electron Corporation), equipped with flame-ionization detector (TRACE GC ULTRA, Thermo Electron Corporation). The conditions were as follows: capillary column (30m x 0,25 mm, Supelcowax 10); column temperatures 160°C (3 min.), 3°C/min to 220°C, 220°C 35 min; carrier gas (18 psi): He, flame-ionization detector. Fatty acids were identified by comparing retention times (*R_t*) with retention time of PUFA standards.

NMR experiments (¹H, ¹³C). NMR analysis were performed on a VarianMercury VX (300 MHz) spectrophotometer. Spectra were obtained in CDCl₃, with TMS as an internal standard.

UPLC-MS analysis. The UPLC-MS/MS system consisted of a Waters ACQUITY® UPLC® (Waters Corporation, Milford, MA, USA) coupled to a Waters TQD mass spectrometer (electrospray ionization mode ESI-tandem quadrupole).

Chromatographic separations were carried out using the Acquity UPLC BEH (bridged ethyl hybrid) C18 column; 2.1 × 100 mm, and 1.7 μm particle size, equipped with Acquity UPLC BEH C18 VanGuard pre-column; 2.1 × 5 mm, and 1.7 μm particle size. The column was maintained at 40°C, and eluted under gradient conditions from 95% to 0% of eluent A over 10 min, at a flow rate of 0.3 mL min⁻¹. Eluent A: water/formic acid (0.1%, v/v); eluent B: acetonitrile/formic acid (0.1%, v/v).

Chromatograms were made using Waters eλ PDA detector. Spectra were analyzed in 200 – 700 nm range with 1.2nm resolution and sampling rate 20 points/s.

MS detection settings of Waters TQD mass spectrometer were as follows: source temperature 150 °C, desolvation temperature 350°C, desolvation gas flow rate 600 L h⁻¹, cone gas flow 100 L h⁻¹, capillary

potential 3.00 kV, cone potential 20 V. Nitrogen was used for both nebulizing and drying gas. The data were obtained in a scan mode ranging from 50 to 1000 m/z in time 0.5 s intervals.

***In vitro* anti-arthritic activity**

Inhibition of albumin denaturation. The anti-inflammatory activity was determined using the inhibition of albumin denaturation technique. The test was performed according to method of Mizushima et al. (Mizushima & Kobayashi 1968) and Williams (Williams et al. 2008) with minor modifications.

An aqueous solution of 0.5 % w/v BSA was prepared and pH was adjusted to 6.0 using 1M HCl. The diclofenac sodium was used as a standard drug. All dry tested extracts, MGDG-1 and DGDG-1 were dissolved in DMSO to obtain stock solutions (5000 µg/mL). These solutions were used to produce final concentrations ranging from 1-500 µg/mL of examined substances. Reagent mixtures, were prepared as follows:

The test solutions (0.5 mL) were prepared by combining 450 µL of aqueous solution of bovine serum albumin (0.5% w/v) and solutions of tested substances (50 µL).

The test control (0.5 mL) consisted of 450 µL of 0.5% (w/v) aqueous solution of bovine serum albumin fraction and DMSO (50 µL).

Product control solutions (0.5 mL) consisted of 450 µL of distilled water and solutions of tested substances (50 µL)

All the reaction mixtures were incubated at 25°C for 20 min and then heated in water bath to 70°C for 5 minutes to denature proteins. After cooling the samples, the turbidity was measured spectrophotometrically at 660 nm (Multi-Detection Microplate Reader SynergyTM HT – BioTek) The experiment was performed in triplicate. The inhibition of albumin denaturation was expressed as percent of inhibition of protein denaturation, relative to the control, which represents 100 % of protein denaturation and was calculated by using the following formula:

$$\% \text{ inhibition} = 100 - [(A_{TS} - A_{PC}) / A_{TC}] \times 100$$

whereas:

A_{TS} - absorbance of the test solution

A_{PC} - absorbance of the test control

A_{TC} - absorbance of the product control solution

The results were compared with diclofenac sodium, a standard anti-inflammatory drug used for arthritis pain.

Anti-hyaluronidase activity. The ability of the extracts to inhibit hyaluronidase was determined by the modified, spectrophotometric method of Yus et al. (Yus & Mashitah 2012). The activity was determined on the basis of precipitation of undigested HA with albumin. The extracts, MGDG-1 and DGDG-1 concentration was 1.0 mg/mL in 10% water ethanol solution. 50 µL of enzyme (30 U/mL of acetate buffer pH 4.5), 50 µL of sodium phosphate buffer (50 mM, pH 7.0; with 77 mM NaCl and 1 mg/mL of albumin) and solutions of the examined substances (11, 22 or 50 µL) were combined in order to produce reagent mixtures having a final concentrations: 68.32, 127.9, 250 µL/ml. All the reaction mixtures were incubated at 37 °C for 10 min. Next, 50 µL of HA (0.3 mg/mL of acetate buffer pH 4.5) was added and incubated at 37 °C for 45 min. The undigested HA was precipitated with 1 mL acid albumin solution made up 0.1% bovine serum albumin in 24 mM sodium acetate and 85 mM acetic acid. The mixture was kept at room temperature for 10 min., the absorbance of the reaction mixture was measured at 600 nm (Multi-Detection Microplate Reader SynergyTM HT – BioTek). Quercetin was used as the positive control, the absorbance in the absence of enzyme was used as the blind control. All assays were performed in triplicates. The percentage of inhibition was calculated as:

$$\% \text{ inhibition} = [(A_B - A_E)/(A_S - A_E)] \times 100$$

whereas:

A_B - absorbance of the enzyme+substrate+ substance sample

A_E - absorbance of the enzyme+substrate sample

A_S - absorbance of the enzyme+ substance sample

Table S1. Results of GLC analysis of fatty acid composition in galactolipids from *I. parviflora* leaves.

Fatty acid	Fatty Acid Composition %	
	MGDGs	DGDGs
10:0	0.038	0.09
12:0	0.153	0.049
14:0	0.248	0.124
14:1	0.029	0.012
15:0	0.142	0.085
16:0	1.648	16.248
16:1 n-9	0.261	0.213
16:1 n-7	0.100	0.537
17:0	0.027	0.097
17:1	0.039	0.034
18:0	0.525	0.746
18:1 n-9	1.199	1.620
18:1 n-7	0.253	0.392
18:2 n-6	1.208	1.547

18:3 n-3	93.935	78.142
20:0	0.029	0.026
20:1	0.016	0.124
$\Sigma_{\text{sat.}}$	2.810	17.465
$\Sigma_{\text{unsat.}}$	97.040	82.621
$\Sigma_{\text{unsat. 18C FA}}$	96.595	81.701

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