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Phytochemical characterization and anti-inflammatory activity of a water extract of *Gentiana purpurea* roots

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ABSTRACT

Ethnopharmacological relevance: Gentiana purpurea was one of the most important medicinal plants in Norway during the 18th and 19th centuries, and the roots were used against different types of gastrointestinal and airway diseases.
Aim of the study: To explore the content of bioactive compounds in a water extract from the roots, a preparation commonly used in traditional medicine in Norway, to assess the anti-inflammatory potential, and furthermore to quantify the major bitter compounds in both roots and leaves.
Materials and methods: G. purpurea roots were boiled in water, the water extract applied on a Diaion HP20 column and further fractionated with Sephadex LH20, reverse phase C18 and normal phase silica gel to obtain the low molecular compounds. 1D NMR, 2D NMR, and ESI-MS were used for structure elucidation. HPLC-DAD analysis was used for quantification. The inhibition of TNF-α secretion in ConA stimulated peripheral blood mononuclear cells (PBMCs) was investigated.
Results: Eleven compounds were isolated and identified from the hot water extract of *G. purpurea* roots. Gentiopicrin, amarogentin, erythrocentaurin and gentiogenal showed dose-dependent inhibition of TNF-α secretion. Gentiopicrin is the major secondary metabolite in the roots, while sweroside dominates in the leaves.
Conclusions: The present work gives a comprehensive overview of the major low-molecular weight compounds in the water extracts of *G. purpurea*, including metabolites produced during the decoction process, and show new anti-inflammatory activities for the native bitter compounds as well as the metabolites produced during preparation of the crude drug.

List of compounds

Compound	CAS RN
Swertiamarin	17388-39-5
Gentiopicroside/gentiopicrin	20831-76-9
Sweroside	14215-86-2
Angelone	904293-35-2
Gentiogenal	87042-24-8
Syringic acid	530-57-4
Erythrocentaurin	50276-98-7
Erythrocentaurin dimethyl acetal	1002101-86-1
Acanthoside B	7374-79-0
Naringenin 4'-O-β-glucopyranoside	81202-36-0
Amarogentin	21018-84-8

1. Introduction

Gentiana purpurea L., family Gentianaceae, is a 20–80 cm high perennial plant with dark purple corollas, and is known for the intensely bitter taste of its roots. It has limited distribution in Europa, growing only in the mountain area in southern parts of Norway and in the Alps (Roskov et al., 2019). *G. purpurea* was regarded as one of the most important medicinal plants in Norway during the 18th and 19th centuries. The roots were used as medicine both for humans and animals and prepared as a water decoction, an alcoholic tincture, boiled in milk, or even in cream or beer (Høeg, 1974). Indications were all kinds of

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List of abbreviations

ConA – Concanavalin A
$CDCl_3$ – deuterated chloroform
CD_3OD – deuterated methanol
DMSO-d_6 – deuterated dimethyl sulfoxide
ESI-MS – electrospray ionization-mass spectrometry
GAE – gallic acid equivalents
HPLC-DAD – high performance liquid chromatography – diode array detector
LOD – limit of detection
LOQ – limit of quantification
PBMC – peripheral blood mononuclear cell
NMR – nuclear magnetic resonance
TNF-α – tumor necrosis factor alpha

stomach diseases, especially diarrhea, but also against colic and to stimulate appetite. The roots were also used against chest diseases such as bronchitis, and to treat coughing and the cold (Ulriksen et al., 2022). The roots were collected during autumn, preferably October, and the dried roots were sold to pharmacies and at local markets, but also exported, e.g. to Sweden (Svanberg, 2002). Due to overextensive and unsustainable harvesting, the plant was eradicated several places in Norway, but is today regarded as a vigorous plant in Norway (Artsdatabanken, 2021).

A few chemical constituents from *G. purpurea* are reported: Secoiridoids identified in the roots are gentiopicrin (also known as gentiopicroside) (Bridel, 1914), desoxyamarogentin (amaropanin) (Wagner and Vasirian, 1974), amarogentin and amaroswerin (Sticher and Meier, 1978, 1980), and gentiolactone (Suhr et al., 1978). An alkaloid, gentianine, was reported to be present in *G. purpurea* (Steinegger and Weibel, 1951). It was later suggested that this is an artefact from treatments of iridoids, such as gentiopicrin and amarogentin, with NH_3 during isolation (Budzikiewicz et al., 1967). The xanthone gentisin is reported from the roots (Verney and Debelmas, 1973). The *C*-glycosylflavones isoorientin, isovitexin and their 4'-glucosides are reported from *G. purpurea* leaves (Hostettmann et al., 1975). The trisaccharide gentianose was first reported in the roots (Meyer, 1882). Other identified carbohydrates are sucrose from the roots (Bridel, 1920), and bornesitol identified in the leaves (Hostettmann and Jacot-Guillarmod, 1974). Also a series of phenolic acids are known from the plant (Dombrowicz and Swiatek, 1987; Hatjimanoli et al., 1988). Reviews of constituents of the genus *Gentiana*, including *G. purpurea*, have been published (Pan et al., 2016; Xu et al., 2017; Yang et al., 2010), however, these reviews lack several of the references mentioned above.

Despite the popularity of this plant in traditional medicine in Norway, no systematic phytochemical characterization of the water extract is reported in the scientific literature. The purpose of the study was to characterize the chemical composition in a root decoction, as this has been a popular preparation in traditional medicine in Norway. Gentiana species are known to have to anti-inflammatory effects (Pan et al., 2016), and we wanted to study anti-inflammatory activities of the decoction and selected isolated constituents to get a deeper insight in how the roots from *G. purpurea* can contribute to medicinal effects. An HPLC-DAD method was developed to quantify the major secoiridoid glucosides in both leaves and roots.

2. Materials and methods

2.1. Plant material

Gentiana purpurea (local name "søterot") (Supplementary Material Fig. S1) was collected in Vang in Valdres, Norway (coordinates 60°59'29.5"N 8°37'41.7"E), 1060 masl, the 13th of August 2020; roots for isolation of constituents, and the 30th of June 2021 (before flowering), the 12th of August 2021 (flowering) and the 8th of October 2021 (after flowering); leaves and roots for quantitative analysis. The identity was verified by botanist Dr. Anneleen Kool, Natural History Museum, University of Oslo, Norway. Voucher specimens, RL-20200813-gp, RL-20210630-gp-r, RL-20210630-gp-l, RL-20210812-gp-r, RL-20210812-gp-l, and RL-20211008-gp-r are deposited at the Department of Pharmacy, University of Oslo, and a herbarium specimen is deposited at the Natural History Museum, University of Oslo. The roots were cleaned and washed, cut in small pieces and air-dried. The leaves were air-dried.

2.2. General methods

1D and 2D NMR spectroscopy was conducted on a Bruker AVIII400 or a Bruker AVII600 instrument (Bruker, Rheinstetten Germany). CD_3OD or $CDCl_3$ was used as solvent with tetramethylsilane as reference (Sigma-Aldrich, St. Louis, MO, USA). HPLC analysis was performed on a LaChrom Elite HPLC system (VWR-Hitachi, Tokyo, Japan) equipped with an L- 2455 diode array detector and a Kinetex C18 100A (150 × 4.6 mm) column (Phenomenex, Torrance, CA, USA). Mass spectra were recorded on a Maxis II-ETD instrument (Bruker), positive or negative mode. Preparative chromatography was performed on a Biotage Select Flash instrument equipped with Biotage Sfär C18 or silica columns (Biotage, Uppsala, Sweden) or with laboratory packed Diaion HP-20 gel (Sigma-Aldrich) or Sephadex LH-20 gel (Pharmacia, Uppsala, Sweden) columns. Preparative HPLC was carried out on a ProStar Polaris system (Varian, Palo Alto, CA, USA) equipped with a Kinetex C18 100A (150 × 21.2, 5 μm) column (Phenomenex), flow rate 15 ml/min. Fractions from CC were combined as indicated by UV-absorbance (Biotage Select flash), or by analytical TLC for open column fractions. Silica gel 60 RP-18 F254S, 0.2 mm thickness foils (Merck, Darmstadt, Germany) were used for TLC, and spots were visualized by UV irradiation (254 and 366 nm), and by spraying with $Ce(SO_4)_2$ (1% in 10% aqueous H_2SO_4) followed by heating (105 °C, 5 min).

2.3. Water extraction

The dried roots were ground in an Ultra Centrifugal Mill ZM 100, 1 mm (Retsch GmbH & Co. KG, Haan, Germany), and 570 g powder was boiled with 5 l distilled water for 1 h. The decoction was centrifuged, plant residue extracted once more under the same conditions and the supernatants combined (crude water extract).

2.4. Isolation of low-molecular compounds

The crude water extract (72% of total amount, 3.6 L) was filtered and applied to a Diaion HP-20 column (42 × 5 cm) eluting with a stepwise gradient of H_2O and methanol to yield fractions D1 (H_2O), D2 (20% methanol), D3 (50% methanol) and D4 (100% methanol). Fraction D2 (500 mg) was applied to a Biotage Sfär C18 column (60 g) and fractioned with a gradient of H_2O and acetonitrile (5–25%), detection by UV absorbance at 250 nm. UV-absorbent fractions were rechromatographed on a Sfär C18 column (12 g) using the same conditions to obtain swertiamarin (**1**) (9 mg), gentiopicrin (**2**) (147 mg) and sweroside (**3**) (14 mg). Fraction D4 (4.5 g) was chromatographed on a Biotage Sfär C18 column (60 g) with a gradient of H_2O and methanol (5–95%), to yield fractions D4F1-D4F9. Fraction D4F1 (47 mg) was chromatographed on a Biotage Sfär C18 column (12 g), UV-detection 270 nm, using a gradient of H_2O and methanol (5–90%) to yield angelone (**4**) (2.3 mg). Fractions D4F2 (217 mg) was applied to a Sephadex LH-20 column (30 × 2 cm) and eluted with a stepwise gradient of H_2O and methanol (25–100%) to give five subfractions. D4F2S3 and S4 were purified on a Biotage Sfär C18 column (12 g), UV detection 270 nm, (gradient H_2O and methanol, 5–90%) to give gentiogenal (**5**) (3.6 mg) and syringic acid (**6**) (1 mg). D4F4 (220 mg) was fractionated on a Biotage Sfär silica column (50 g)

with a stepwise gradient of dichloromethane and ethyl acetate (1:0, 1:9, 1:3, 1:1, 0:1, 2 CV each), 270 nm, to obtain erythrocentaurin (**7**) (44 mg). D4F5 (319 mg) was chromatographed on a Biotage Sfär C18 column (60 g), UV-detection 270 nm, with a gradient of H_2O and methanol (5–95%), to give erythrocentaurin (**7**) (23 mg) and erythrocentaurin dimethylacetal (**8**) (8 mg). The fraction between the peaks representing compounds **7** and **8** (D4F5F2, 117 mg) was further purified on a Biotage Sfär silica column (10 g), UV detection 260 nm, with a gradient of dichloromethane and methanol (5–95%), to give acanthoside B (**9**) (26 mg). D4F8 (989 mg) was chromatographed on a Biotage Sfär C18 column (60 g), UV detection 270 nm, with a gradient of H_2O and methanol (20–95%) to yield four subfractions. Fraction D4F8F2 (31 mg) was rechromatographed on a Biotage Sfär C18 column (60 g) followed by a Biotage Sfär C18 column (12 g) using a H_2O-methanol gradient (20–95%), UV detection 270 nm, to give naringenin 4'-*O*-β-glucopyranoside (**10**) (2.1 mg). D4F8F4 (204 mg) was applied on a Sephadex LH-20 column (30 × 2 cm) and eluted with a step wise gradient of H_2O and methanol (25–100%) and yielded amarogentin (**11**) (69 mg). NMR spectra of compounds **1**–**11** are shown in supplementary material, Figs. S3–S13.

Compound **1** (swertiamarin, $C_{16}H_{22}O_{10}$)

ESI-MS (+) m/z 397.11 $[M+Na]^+$

^1H NMR (400 MHz, CD_3OD) δ 7.63 (s, 1H, H-3), 5.72 (d, *J* = 1.4 Hz, 1H, H-1), 5.44 (m, 1H, H-8), 5.36 (dd, *J* = 17.0, 2.5 Hz, 1H, H-10), 5.29 (dd, *J* = 9.5, 2.5 Hz, 1H, H-10), 4.75 (ddd, *J* = 12.8, 10.9, 2.7 Hz, 1H, H-7), 4.64 (d, *J* = 7.9 Hz, 1H, H-1'), 4.34 (ddd, *J* = 10.9, 5.1, 1.7 Hz, 1H, H-7), 3.89 (dd, *J* = 12.0, 2.1 Hz, 1H, H-6'), 3.67 (dd, *J* = 11.9, 5.6 Hz, 1H, H-6'), 3.26–3.40 (overlapping signals, H-3', H-4' and H-5'), 3.21 (dd, *J* = 9.1, 7.9 Hz, 1H, H-2'), 2.92 (dd, *J* = 9.2, 1.4 Hz, 1H, H-9), 1.91 (ddd, *J* = 14.1, 12.8, 5.1 Hz, 1H, H-6), 1.75 (brd, J = 14.3, 1H, H-6), in accordance with (Li et al., 2015); ^{13}C NMR (100 MHz, CD_3OD) δ 168.03 (C-11), 154.82 (C-3), 133.85 (C-8), 121.24 (C-10), 108.89 (C-4), 100.26 (C-1'), 99.13 (C-1), 78.54 (C-5'), 77.82 (C-3'), 74.44 (C-2'), 71.43 (C-4'), 65.99 (C-7), 64.31 (C-5), 62.60 (C-6'), 51.94 (C-9), 33.75 (C-6), in accordance with (Boros and Stermitz, 1991).

Compound **2** (gentiopicrin, $C_{16}H_{20}O_9$)

ESI-MS (+) m/z 379.10 $[M+Na]^+$

^1H NMR (400 MHz, CD_3OD) δ 7.47 (d, *J* = 1.5 Hz, 1H, H-3), 5.78 (ddd, *J* = 17.2, 10.3, 6.9 Hz, 1H, H-8), 5.68 (d, *J* = 2.9 Hz, 1H, H-1), 5.64 (m, 1H, H-6), 5.26 (m, 1H, H-10), 5.22 (dt, *J* = 10.3, 1.2 Hz, 1H, H-10), 5.09 (m, 1H, H-7), 5.01 (m, 1H, H-7), 4.67 (d, *J* = 7.9 Hz, 1H, H-1'), 3.92 (dd, *J* = 11.9, 2.2 Hz, 1H, H-6'), 3.67 (dd, *J* = 11.9, 6.1 Hz, 1H, H-6'), 3.27–3.41 (overlapping signals, H-9, H-3', H-4' and H-5'), 3.18 (dd, *J* = 9.2, 7.9 Hz, 1H, H-2'), in accordance with (Li et al., 2015); ^{13}C NMR (100 MHz, CD_3OD) δ 166.30 (C-11), 150.65 (C-3), 135.01 (C-8), 127.01 (C-5), 118.54 (C-10), 117.20 (C-6), 104.94 (C-4), 100.19 (C-1'), 98.52 (C-1), 78.40 (C-5'), 77.96 (C-3'), 74.54 (C-2'), 71.52 (C-4'), 70.91 (C-7), 62.77 (C-6'), 46.60 (C-9), in accordance with (Boros and Stermitz, 1991).

Compound **3** (sweroside, $C_{16}H_{22}O_9$)

ESI-MS (+) m/z 381.12 $[M+Na]^+$

^1H NMR (400 MHz, CD_3OD) δ 7.59 (d, *J* = 2.5 Hz, 1H, H-3), 5.55 (m, 1H, H-8), 5.55 (d, *J* = 1.7 Hz, 1H, H-1), 5.31 (m, 2H, H-10), 5.27 (m, 1H, H-10), 4.68 (d, *J* = 7.9 Hz, 1H, H-1'), 4.45 (ddd, *J* = 11.1, 4.3, 2.2 Hz, 1H, H-7), 4.37 (td, *J* = 11.5, 2.8 Hz, 1H, H-7), 3.89 (dd, *J* = 11.9, 2.1 Hz, 1H, H-6), 3.66 (dd, *J* = 11.9, 5.7 Hz, 1H, H-6), 3.19 (dd, *J* = 9.2, 7.9 Hz, 1H, H-2'), 3.28–3.96 (overlapping signals, H-5, H-3', H-4' and H-5'), 2.70 (ddd, *J* = 9.7, 5.5, 1.8 Hz, 1H, H-9), 1.77 (m, 1H, H-6), 1.70 (m, 1H, H-6), in accordance with (Li et al., 2015).

Compound **4** (angelone, $C_9H_8O_4$)

ESI-MS (+) m/z 203.03 $[M+Na]^+$

^1H NMR (400 MHz, CD_3OD) δ 8.40 (s, 1H, H-7), 4.54 (t, *J* = 6.0 Hz, 2H, H-3), 3.19 (t, *J* = 6.0 Hz, 2H, H-4), 2.50 (s, 3H, H-11), in accordance with (Mulholland et al., 2006).

Compound **5** (gentiogenal, $C_{10}H_{10}O_4$)

ESI-MS (+) m/z 217.05 $[M+Na]^+$

^1H NMR (400 MHz, $CDCl_3$) δ 9.88 (s, 1H, H-11), 7.94 (s, 1H, H-8), 5.64 (q, *J* = 6.5 Hz, 1H, H-6), 4.41 (m, 2H, H-3), 3.08 (m, 2H, H-4), 1.40 (d, *J* = 6.5 Hz, 3H, H-12);

^{13}C NMR (100 MHz, $CDCl_3$) δ 185.43 (C-11), 163.2 (C-1), 163.18 (C-8), 142.50 (C-10), 120.1 (C-5), 104.3 (C-9), 73.28 (C-6), 65.04 (C-3), 22.81 (C-4), 19.84 (C-12), in accordance with (Boros and Stermitz, 1991).

Compound **6** (syringic acid, $C_9H_{10}O_5$)

ESI-MS (+) m/z 221.04 $[M+Na]^+$

^1H NMR (400 MHz, CD_3OD) δ 7.33 (s, 2H, H-2 and H-6), 3.88 (s, 6H, 3-OCH_3 and 5-OCH_3), compared with standard compound (Fluka, Buchs, Switzerland).

Compound **7** (erythrocentaurin, $C_{10}H_8O_3$)

ESI-MS (+) m/z 199.04 $[M+Na]^+$

^1H NMR (400 MHz, $CDCl_3$) δ 10.23 (s, 1H, H-11), 8.38 (dd, *J* = 7.8, 1.5 Hz, 1H, H-8), 8.07 (dd, *J* = 7.6, 1.5 Hz, 1H, H-6), 7.64 (t, *J* = 7.7 Hz, 1H, H-7), 4.57 (t, *J* = 6.1 Hz, 2H, H-3), 3.59 (t, *J* = 6.1 Hz, 2H, H-4); ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.86 (C-11), 164.16 (C-1), 141.14 (C-10), 138.38 (C-8), 135.61 (C-5), 132.61 (C-6), 127.86 (C-9), 126.92 (C-7), 66.74 (C-3), 24.60 (C-4), in accordance with (Wang et al., 2009).

Compound **8** (erythrocentaurin dimethylacetal, $C_{12}H_{14}O_4$)

ESI-MS (+) m/z 245.08 $[M+Na]^+$

^1H NMR (400 MHz, $CDCl_3$) δ 8.13 (dd, *J* = 7.9, 1.4 Hz, 1H, H-8), 7.77 (dd, *J* = 7.7, 1.4 Hz, 1H, H-6), 7.41 (t, *J* = 7.7 Hz, 1H, H-7), 5.43 (s, 1H, H-11), 4.51 (t, *J* = 6.1 Hz, 2H, H-3), 3.32 (s, 6H, H-12 and H-13), 3.16 (t, *J* = 6.1 Hz, 2H, H-4), in accordance with (Ando et al., 2007).

Compound **9** (acanthoside B, $C_{28}H_{36}O_{13}$)

ESI-MS (+) m/z 603.21 $[M+Na]^+$

^1H NMR (400 MHz, CD_3OD) δ 6.71 (s, 2H, H-2 and H-6), 6.65 *(*s, 2H, H-2' and H-6'), 4.85 (overlapped), 4.76 (d, *J* = 4.0 Hz, 1H, H-7), 4.76 (d, *J* = 4.2 Hz, 1H, H-7'), 4.28 (m, 2H, H-9_b and H-9'$_b$), 3.90 (m, 2H, H-9_a and H-9'$_a$), 3.86 (s, 6H, 3-OCH_3 and 5-OCH_3), 3.84 (s, 6H, 3'-OCH_3 and 5'-OCH_3), 3.77 (*dd*, *J* = 12.1, 2.6 Hz, 1H, H-6''$_b$), 3.66 (*dd*, *J* = 12.1, 5.2 Hz, 1H, H-6''$_a$), 3.47 (m, 1H, H-2''), 3.41 (m, 2H, H-3'' and H-4''), 3.20 (*m* , 1H, H-5''), 3.13 (m, 2H, H-8 and H-8');

^{13}C NMR (100 MHz, CD_3OD) δ 154.44 (C-3 and C-5), 149.37 (C-3' and C-5'), 139.57 (C-1), 136.23 (C-4'), 135.60 (C-4), 133.10 (C-1'), 105.36 (C-1''), 104.85 (C-2 and C-6), 104.53 (C-2' and C-6'), 87.62 (C-7'), 87.21 (C-7), 78.36 (C-5''), 77.85 C-3''), 75.73 (C-2''), 72.92 (C-9), 72.95 (C-9'), 71.35 (C-4''), 62.60 (C-6''), 57.10 (3-OCH_3 and 5-OCH_3), 56.84 (3'-OCH_3 and 5'-OCH_3), 55.75 (C-8'), 55.54 (C-8), in accordance with (Shahat et al., 2004).

Compound **10** (naringenin 4'-*O*-β-glucopyranoside, $C_{21}H_{22}O_{10}$)

ESI-MS (+) m/z 457.11 $[M+Na]^+$

^1H NMR (400 MHz, CD_3OD) δ 7.42 (d, *J* = 8.6 Hz, 1H, H-2' and H-6'), 7.14 (d, *J* = 8.6 Hz, 1H, H-3' and H-5'), 5.90 (d, *J* = 2.1 Hz, 1H, H-6), 5.88 (d, *J* = 2.1 Hz, 1H, H-8), 5.40 (dd, *J* = 12.6, 3.1 Hz, 1H, H-2), 4.94 (d, *J* = 7.2 Hz, 1H, H-1''), 3.90 (dd, *J* = 12.0, 2.1 Hz, 1H, H-6''), 3.70 (dd, *J* = 12.0, 5.4 Hz, 1H, H-6''), 3.40–3.50 (overlapping signals, H-2'', H-3'', H-4'' and H-5''), 3.09 (dd, *J* = 17.1, 12.6 Hz, 1H, H-3_a), 2.73 (dd, *J* = 17.1, 3.1 Hz, 1H, H-3_b);

^{13}C NMR (100 MHz, CD_3OD) δ 197.41 (C-4), 168.61 (C-9), 165.47 (C-7), 164.69 (C-5), 159.27 (C-4'), 134.19 (C-1'), 128.79 (C-2' and C-6'), 117.80 (C-3' and C-5'), 103.32 (C-10), 102.18 (C-1''), 97.18 (C-8), 96.27 (C-6), 80.12 (C-2), 78.07 (C-5''), 77.97 (C-3''), 74.89 (C-2''), 71.36 (C-4''), 62.50 (C-6''), 44.03 (C-3), in accordance with (da Silva et al., 2013).

Compound **11** (amarogentin, $C_{29}H_{30}O_{13}$)

ESI-MS (+) m/z 609.16 $[M+Na]^+$

^1H NMR (600 MHz, CD_3OD) δ 7.43 (d, *J* = 2.6 Hz, 1H, H-3), 7.17 (t, *J* = 8.0 Hz, 1H, H-5'''), 6.78 (ddd, *J* = 8.0, 2.4, 1.1 Hz, 1H, H-4'''), 6.70–6.74 (overlapping signals, 2H, H-2''' and H-6'''), 6.30 (d, *J* = 2.4 Hz, 1H, H-4''), 6.17 (d, *J* = 2.4 Hz, 1H, H-6''), 5.44 (dt, *J* = 17.1, 9.7 Hz, 1H, H-8), 5.39 (d *J* = 1.8, 1H, H-1), 5.24 (m, 2H, H-10) 4.73 (dd, *J* = 9.4, 8.0 Hz, 1H, H-2'), 4.37 (m, 1H, H-7), 4.29 (d, *J* = 8.0 Hz, 1H, H-1'), 4.25 (m, 1H, H-7), 3.84 (dd, *J* = 12.1, 2.2 Hz, 1H, H-6'), 3.61 (dd, *J* = 12.1, 6.2

Hz, 1H, H-6'), 3.23 (t, $J = 9.3$ Hz, 1H, H-4'), 3.09 (m, 1H, H-5'), 2.82 (t, $J = 9.3$ Hz, 1H, H-3'), 2.74 (m, 1H, H-5), 2.58 (ddd, $J = 9.6$, 5.5, 1.8 Hz, 1H, H-9), 1.69 (m, 1H, H-6), 1.58 (qd, $J = 12.8$, 4.2 Hz, 1H, H-6); ^{13}C NMR (150 MHz, CD_3OD) δ 171.44 (CO), 167.58 (C-11), 165.97 (C-3''), 163.86 (C-5''), 157.41 (C-3'''), 153.69 (C-3), 148.58 (C-1''), 146.48 (C-1'''), 132.80 (C-8), 129.31 (C-5'''), 121.15 (C-6'''), 121.00 (C-10), 116.48 (C-2'''), 114.53 (C-4'''), 112.85 (C-6''), 105.57 (C-4), 104.04 (C-2''), 103.14 (C-4''), 97.16 (C-1), 96.76 (C-1'), 78.31 (C-5'), 74.82 (C-3'), 74.60 (C-2'), 71.63 (C-4'), 69.51 (C-7), 62.42 (C-6'), 43.38 (C-9), 28.67 (C-5), 25.78 (C-6), in accordance with (Wang et al., 2001).

2.5. Quantitative analysis of secoiridoid glucosides with HPLC-DAD

The dried roots were ground in a knife mill (Brabender, Duisburg, Germany; 4 mm sieve). Leaves were pulverized using mortar and pistil. Analytical standards of gentiopicrin and sweroside were obtained from Sigma-Aldrich (≥99% purity), the purity of isolated amarogentin was determined by HPLC (250 nm, > 99% purity) (supplementary material, Fig. S2).

Methanol extracts were obtained by extracting 25.0 mg powdered roots or leaves in 25 mL methanol in a Falcon tube on ultrasonic bath for 40 min. The extract was centrifuged at 4000 RPM for 10 min, the supernatant was decanted, and the extraction step repeated. The supernatants were filtered through an PTFE syringe filter (0.45 μm) into an accurately weighed round-bottomed flask and dried on a rotavapor. Methanol was added to give a final concentration of 2.0 mg/mL, and the solution was diluted 1:1 with distilled water before HPLC analysis. Elution was performed using a gradient of mobile phase A (H_2O) and B (acetonitrile) with the following time schedule: 5% B, 0–3 min; 5–25% B, 3–20 min; 25–90% B, 20–45 min; 90% B, 45–50 min. The flow rate was 1 ml/min, injection volume 10 μL, temperature 25 °C, and absorbance was recorded at 225, 246 and 275 nm. Quantification was based on individual standard curves for each analyte. Gentiopicrin, sweroside and amarogentin were accurately weighed, dissolved in methanol and diluted to appropriate concentrations. Standard curves were based on three parallels with eight different concentrations, and UV maximum for each standard employed; gentiopicrin 275 nm, sweroside 246 nm, and amarogentin 225 nm (Table 1). The sample solutions were filtered through a PTFE syringe filter (0.45 μm) and analyzed in triplicate. The results are expressed as mg substance per gram dry weight.

2.6. Total phenolic content

The total phenolic content of roots and leaves collected at different dates was measured by use of the Folin-Ciocalteu method as previously described (Ulriksen et al., 2022) using a SpectraMax 190 Microplate Reader (Molecular Devices, San Jose, CA, USA). The results are expressed as mg gallic acid equivalents (GAE) per gram dry weight.

Table 1
Calibration curve, LOD and LOQ for standard compounds.

Standard	Calibration curve	R^2	Concentration range (μg/mL)[a]	LOD (μg/mL)[b]	LOQ (μg/mL)[c]
Gentiopicrin (**2**)	y = 62377.7x + 10806.7	0.9999	1–200	0.31	0.94
Sweroside (**3**)	y = 75695.3x + 79627.7	0.9996	1–200	2.3	6.9
Amarogentin (**11**)	y = 92161.8x + 28187.3	0.9999	0.5–100	0.29	0.88

[a] Based on 8 different concentrations.
[b] LOD (limit of detection), 3.3 × standard deviation of the y-intercepts of regression line/slope of the regression line (σ/S).
[c] LOQ (limit of quantification), 10 × standard deviation of the y-intercepts of regression line/slope of the regression line (σ/S).

2.7. Anti-inflammatory assay

Release of human TNF-α ELISA was tested from human peripheral blood mononuclear cells (PBMCs), using an ELISA kit from Mabtech (Sweden) as described previously (Ulriksen et al., 2022). PBMCs were isolated via Lymphoprep (StemCell Technologies, Vancouver, Canada) from buffy coats obtained from healthy volunteers at the Blood Bank at Oslo University Hospital (the use of PBMCs are approved by the Regional Ethical Committee). In brief, PBMCs were incubated with indicated concentration of substances overnight at 37 °C in a 5% CO_2 cell incubator. In each well, 0.5% DMSO were spiked in to ensure equal DMSO concentration in all wells. 10 ng/mL concanavalin A (ConA) were used as an inducer of TNF-α release. Cell-free culture supernatants (100 μL) and standards were added to 96-well plates pre-coated with capture antibodies and blocked in PBS with 0.05% Tween-20 and 0.1% BSA. After 2 h incubation, the plates were washed and 100 μL/well of human TNF-α monoclonal detection antibody (Mabtech) diluted in incubation buffer (1 μg/mL) were added, plates were incubated at 1 h in room temperature (RT). After washing, streptavidin-HRP (Mabtech) were added, and plates were incubated for 1 h at RT. Plates were developed with TMB substrate for 15 min followed by 1 M HCl. Absorbance at 450 nm was measured using a Molecular Devices FlexStation 3 Reader within 15 min of adding HCl. Data are calculated based on standard curve and presented as percent inhibition based on the ConA alone and DMSO alone. All samples were run in duplicates on the same plate and all runs were repeated with three different donors. All washes were done using a BioTek ELx405 plate washer with 0.05% Tween-20 in PBS.

2.8. Statistics

Statistical analysis was conducted by using the GraphPad Prism 9 software (GraphPad). Analysis was done by ordinary one-way ANOVA test, Tukey's multiple comparison test was used for comparison of secoiridoid and total phenolic content among the samples, while Dunnett's test for comparison of TNF-α secretion against the untreated control. Values are expressed as mean ± SD.

3. Results and discussion

3.1. Phytochemical composition

Eleven low molecular weight compounds were isolated from the hot water extract of *G. purpurea* roots. Their chemical structures are shown in Fig. 1. The structures were identified by 1D and 2D NMR spectroscopy, comparison of their spectroscopic data with literature values, and the structures confirmed with mass spectrometry (ESI-MS). The isolated compounds include the secoiridoid glucosides swertiamarin (**1**) (Boros and Stermitz, 1991; Li et al., 2015), gentiopicrin (**2**) (Boros and Stermitz, 1991; Li et al., 2015), sweroside (**3**) (Li et al., 2015) and amarogentin (**11**) (Wang et al., 2001), the secoiridoids angelone (**4**) (Mulholland et al., 2006), gentiogenal (**5**) (Boros and Stermitz, 1991), erythrocentaurin (**7**) and erythrocentaurin dimethylacetal (**8**) (Ando et al., 2007), the lignan glucoside acanthoside B (**9**) (Shahat et al., 2004), and the flavanone naringenin 4'-*O*-β-glucopyranoside (**10**) (da Silva et al., 2013). Isolated syringic acid (**6**) was identified by comparison with NMR spectra of reference compound obtained from Fluka. Only gentiopicrin, amarogentin, and syringic acid were previously known in this species (Bridel, 1920; Dombrowicz and Swiatek, 1987; Nyiredy et al., 1986; Sticher and Meier, 1978, 1980). Swertiamarin and sweroside are well known from the *Gentiana* genus (Pan et al., 2016), while acanthoside B and naringenin 4'-*O*-β-glucoside have not been reported from the genus before, and the finding in this taxon is therefore of chemotaxonomic interest. Interestingly, flavanones are rare compounds among *Gentiana* species, and only a few flavanones seems to be reported from this taxon

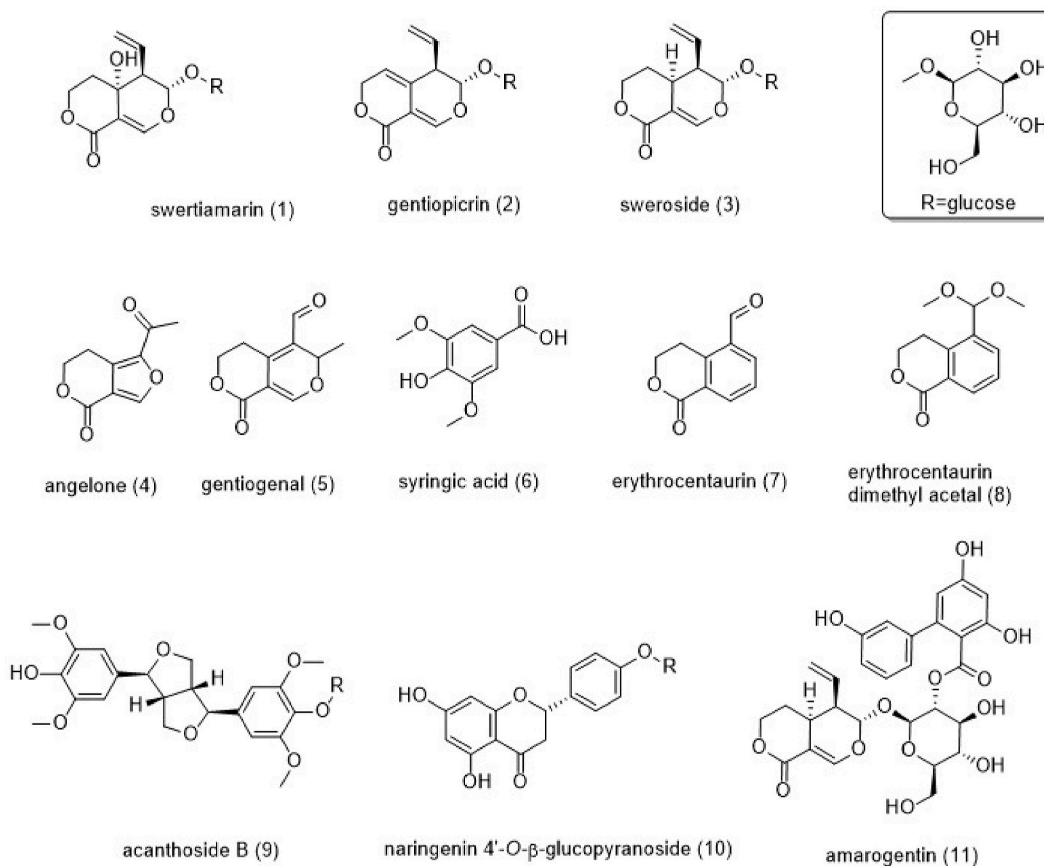

Fig. 1. Chemical structures of compounds **1**–**11** isolated from a water extract of *Gentiana purpurea* roots.

(Pan et al., 2016; Xu et al., 2017; Yang et al., 2010). Gentiogenal and erythrocentaurin were probably formed during hot water extraction. Gentiogenal and erythrocentaurin contain an aldehyde group giving rise to characteristic ^{1}H NMR signals at δ 9.83 ppm (s) and 10.24 ppm (s), respectively, in the water extract (^{1}H NMR, DMSO-d6). These signals were not present in a methanol extract of the gentian roots, and the difference in gentiogenal and erythrocentaurin content in the water and methanol extracts were also verified by HPLC-DAD analysis (Fig. 2). During purification of erythrocentaurin by RP-C18 flash chromatography with a water-methanol gradient, erythrocentaurin dimethylacetal appeared as a new compound and was not present in the crude extract. Erythrocentaurin and gentiogenal are previously reported as degradation products from gentiopicrin, which supports the findings in this study that these molecules are degradation products formed during boiling with water (El-Sedawy et al., 1989; Ishiguro et al., 1983; Wang et al., 2009). The pH of the water was 4.1, which may contribute to an acidic hydrolysis of the glycosidic bond of the iridoid glucosides. However, enzymatic degradation during heating with water cannot be excluded and will be a subject for a follow up study.

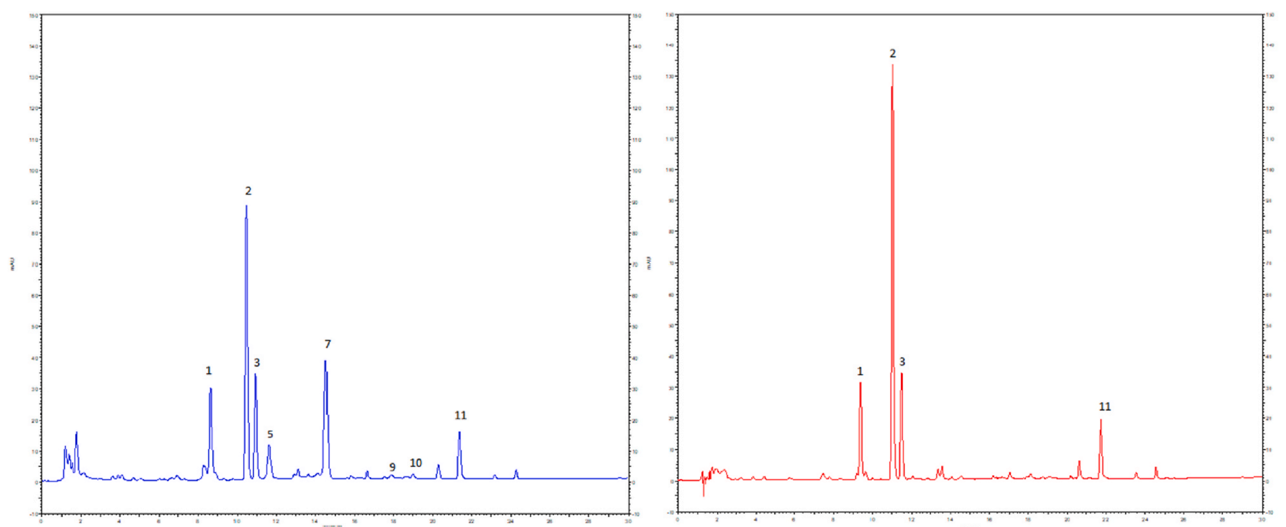

Fig. 2. HPLC chromatograms at 225 nm of *Gentiana purpurea* root water extract (left) and methanol extract (right).

3.2. Quantitative analysis

Significant differences between the content of gentiopicrin, sweroside, and amarogentin in leaves and roots were observed (Table 2). Gentiopicrin is the major metabolite in the roots (49.7–67.6 mg/g), while sweroside is the major metabolite in the leaves (26.4–26.6 mg/g). Amarogentin was not detected in the leaves. Highest yield of the three analyzed secoiridoids was obtained with roots collected in October, while no significant differences were observed for the secoiridoid content in the leaves collected at different time points. The quantitative content of gentiopicrin and amarogentin is comparable with the values reported in the roots of *G. purpurea* (Sticher and Meier, 1980), 33–100 mg/g for gentiopicrin and 1.86–5.10 mg/g for amarogentin. However, the analysis from 1980 did not distinguish between gentiopicrin and sweroside, which elute quite close to each other on the RP18 column. This is the first report of the identification and quantification of gentiopicrin, sweroside, and amarogentin in *G. purpurea* leaves.

The phenolic content, measured as GAE, was higher in the leaves than in the roots. This is in accordance with other *Gentiana* studies, e.g. (Stefanović et al., 2018). There were only minor differences between the samples collected at different dates. In Norway, *G. purpurea* roots were commonly harvested from August to October, preferably in late autumn. This study supports the local harvesting tradition with a preference for harvesting in October, since a high content of bitter substances is wanted.

3.3. Anti-inflammatory effects

Gentiopicrin (**2**), gentiogenal (**5**), erythrocentaurin (**7**), amarogentin (**11**), and the crude water extract were evaluated for immune inhibitory effects by measuring their capacity to reduce TNF-α secretion in ConA stimulated PBMCs. The tested compounds showed a significant dose-dependent anti-inflammatory effect, with small differences between the four compounds (Fig. 3). TNF-α inhibition was slightly stronger for erythrocentaurin (39.5%) compared to gentiopicrin (27.9%), gentiogenal (27.1%) and amarogentin (27.9%) at the lowest concentration (12.5 μM). The same trend was observed at 25 μM, with 58.3% inhibition for erythrocentaurin compared to 49.0% (gentiopicrin), 46.4% (gentiogenal) and 53.0% (amarogentin). The crude water extract (12.5–100 μg/mL) showed significant inhibitory effects (15.8–26.0% inhibition), but no clear dose dependency was observed. This is probably caused by the high amounts of carbohydrates in the *G. purpurea* water extract, since a polysaccharide enriched fraction from *G. purpurea* has shown an opposite effect with stimulation of TNFα production (Ulriksen et al., 2022).

Previous studies have reported anti-inflammatory effects of gentiopicrin (Jia et al., 2022; Kondo et al., 1994; Wang et al., 2013) and amarogentin (Huang et al., 2020b; Potunuru et al., 2019; Wölfle et al., 2015). However, this is the first study demonstrating a reduction of TNF-α secretion in PBMCs of these two compounds. Very few biological

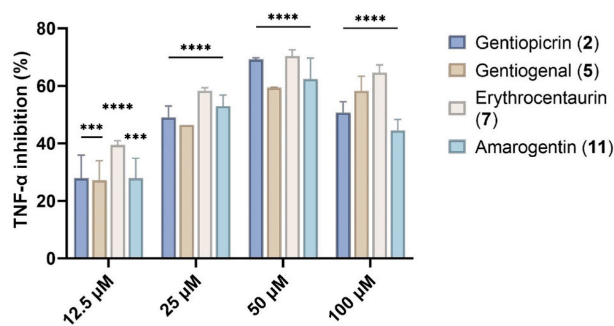

Fig. 3. Anti-inflammatory effects of isolated compounds presented as % inhibition of TNF-α in peripheral blood mononuclear cells (PBMCs) compared to ConA stimulated cells (untreated). Each bar express the average of two individual experiments ±SD. **** = $p < 0.0001$, *** = $p < 0.001$ compared to control cells (ConA + DMSO).

studies have previously been performed on gentiogenal and erythrocentaurin. This is the first study showing anti-inflammatory activity of gentiogenal, while erythrocentaurin was found to inhibit NO formation in macrophages (Huang et al., 2020a).

4. Conclusions

This study has explored the chemical composition of a hot water extract from *G. purpurea*, one of the most important and popular medicinal plants in Norway during the 18th and 19th centuries. It includes the finding of low molecular compounds new to this plant in the water extract and the quantification of the major secoiridoid glycosides in roots and leaves collected at different dates. For the first time we report the formation of degradation products in the decoction of *G. purpurea*, with erythrocentaurin (**7**) as the major of these products. Extraction with boiling water can result in chemical modifications of the plant metabolites resulting in new metabolites. There is therefore a potential in the area of traditional medicine research to explore new bioactive compounds developed during different preparation processes. Anti-inflammatory activities, observed as reduction in TNF-α secretion in PBMCs, were observed for the major bitter compounds; gentiopicrin (**2**) and amarogentin (**11**), as well as the degradation products; gentiogenal (**5**) and erythrocentaurin (**7**), with erythrocentaurin showing the strongest effect. Thus, the study has contributed to a better understanding of the traditional use, phytochemical profile and biological properties of a hot water extract obtained from *G. purpurea*.

CRediT authorship contribution statement

Lin Zhang: Formal analysis, Data curation, Writing – review & editing. **Emilie Steinbakk Ulriksen:** Formal analysis, Data curation,

Table 2
Content of gentiopicrin, sweroside, and amarogentin in *Gentiana purpurea* roots and leaves (dry weight) collected at three different dates.

Sample, collection date	Voucher specimen	Gentiopicrin (mg/g ± SD)	Sweroside (mg/g ± SD)	Amarogentin (mg/g ± SD)	Total phenolic content (mg GAE/g ± SD) [a]
Roots, 30.06.2021	RL-20210630-gp-r	57.4 ± 0.85[a]	17.8 ± 0.34[a]	2.9 ± 0.040[a]	10.5 ± 1.3[a]
Roots, 12.08.2021	RL-20210812-gp-r	49.7 ± 0.68[b]	11.8 ± 0.15[b]	2.7 ± 0.027[b]	8.4 ± 1.3[ab]
Roots, 08.10.2021[2]	RL-20211008-gp-r	67.7 ± 0.98[c]	18.2 ± 0.33[a]	3.6 ± 0.047[c]	7.1 ± 0.9[b]
Leaves, 30.06.2021	RL-20210630-gp-l	4.2 ± 0.0083[d]	26.4 ± 0.59[c]	< LOD	17.9 ± 3.1[c]
Leaves, 12.08.2021	RL-20210812-gp-l	3.9 ± 0.049[d]	26.6 ± 0.36[c]	< LOD	19.1 ± 2.9[c]

[2]Leaves were withered and not possible to obtain for analyses at this date. Different letters shown in superscript indicate statistical significant differences between groups according to the Tukey's multiple comparison test, $p < 0.05$; n = 3 for gentiopicrin, sweroside and amarogentin, n = 6 for total phenolic content.
[a] GAE; gallic acid equivalents.

Writing - original draft, Writing - review & editing. **Håvard Hoel:** Formal analysis, Data curation, Writing – review & editing. **Lene Sandvik:** Formal analysis, Data curation, Writing – review & editing. **Karl Egil Malterud:** Formal analysis, Data curation, Writing – review & editing. **Kari Tvete Inngjerdingen:** Formal analysis, Data curation, Funding acquisition, Project administration, Writing - review & editing. **Marit Inngjerdingen:** Formal analysis, Data curation, Funding acquisition, Project administration, Writing - review & editing. **Helle Wangensteen:** Formal analysis, Data curation, Funding acquisition, Project administration, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2022.115818.

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