SUPPLEMENTARY MATERIAL

Secondary Metabolites from *Gentiana cruciata* L. and Their Anti-Inflammatory and Analgesic Activities

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ABSTRACT

A previously unreported secoiridoid glycoside, cruciatoside (**1**) was isolated from the aerial parts of *Gentiana cruciata* L. along with ten known compounds eustomoside (**2**), eustomorusside (**3**), gentiopicroside (**4**), 6'-*O*-β-D-glucopyranosyl gentiopicroside (**5**), loganic acid (**6**), isoorientin (**7**), isovitexin (**8**), isovitexin 2"-*(E)*-ferulate (**9**), mangiferin (**10**), and 2-methyl-inositol (**11**). The chemical structures of the isolates were elucidated based on extensive 1D and 2D NMR experiments as well as HRMS analysis. All isolates were evaluated for their *in vitro* anti-inflammatory and analgesic activities. Compounds **9**, **4**, and **7** (200 μM) showed moderate anti-inflammatory activity by inhibiting nitrite production from LPS-induced RAW 264.7 macrophage cells, with the inhibition rates of 39.5%, 25.8% and 22.9% respectively without exhibiting substantial cytotoxicity. Besides, **1**, **2**, **4**, and **7** exerted the highest decrease in IL-6 levels. Moreover, compound **4** showed *in vitro* analgesic activity by decreasing the PGE_2 level comparable to the reference drugs.

Keywords: *Gentiana cruciata*; Gentianaceae; Secoiridoid; Cruciatoside; Anti-inflammatory and analgesic activity

CONTENTS

General experimental procedures

UV and IR spectra were recorded on a HP Agilent 8453 spectrophotometer and a Perkin-Elmer 2000 FT-IR spectrometer, respectively. NMR experiments were performed on a Bruker Avance DRX 500 spectrometer at 400 MHz for ^{1}H and 100 MHz for ^{13}C. NMR spectra were recorded in deuterated methanol (CD_3OD) and solvent signals were taken as references. The chemical shift values (d) were presented in ppm and coupling constants (J) are in Hz. HRMS data were measured on Agilent 6200 series TOF/6500 series mass spectrometer. For medium-pressure liquid chromatographic (MPLC) separations, the SepacoreVR Flash Systems X10/X50 (Buchi) system was used with RediSep columns (LiChroprep C_{18}: 130, 50, and 43 g; SiO_2: 40 g, 24 g, and 12 g; Teledyne Isco). Sephadex LH-20 (Fluka) was used for gel filtration chromatography whereas open column chromatography (CC) was performed with SiO_2 (Merck). Fractions were monitored on silica gel 60 F_{254} precoated TLC plates (Merck) and detected by 1% vanillin/H_2SO_4. The solvents used for chromatographic separations were of analytical grade.

Cytotoxicity Assay

The cytotoxicity of the isolates was evaluated on RAW 264.7 murine macrophage cell line obtained from the American Type Culture Collection (ATCC) was cultured in DMEM (10% FBS, v/v; Gibco) and supplemented with 1% antibiotics (10.000 µg/mL streptomycin and 10.000 units/mL penicillin; Gibco) at 37°C in a humidified atmosphere of 5% CO_2. Prior to the assessment of anti-inflammatory and analgesic activities of compounds isolated from *Gentiana cruciata* (GCR), cytotoxicity profiles of compounds have been assessed via MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; Sigma-Aldrich) test. Briefly, RAW 264.7 cells were plated in 48-well plates and incubated for 24 h at 37°C in 5% CO_2 to form a confluent layer. After 24 h of incubation, cells were treated with serial dilutions of compounds at final concentrations between 25-200 µM. Following the 24 h of incubation, the cell culture medium was discarded and MTT (0.5 mg/mL) was added to each well for an additional 2 h at 37°C. Following the incubation, the medium was discarded and produced formazan crystals were dissolved by 100 µL of isopropanol. The optical density was measured at 570 nm wavelengths using a microplate reader (Multiskan Ascent; ThermoFischer). The percentage of cell viability was detected by using the following equation (Sipahi et al. 2022):

$$\text{Viability \%} = [(\text{Absorbance})_{\text{treatment group}} - (\text{Absorbance})_{\text{control}}] \times 100\%$$

Anti-inflammatory Activity

Nitrite assay

The anti-inflammatory activity of compounds was determined by Griess assay as previously performed in our study (Erdoğan et al. 2021). Briefly, RAW 264.7 cells were plated in a 48-well plate and incubated for 24 h at 37° C in 5% CO_2. The following day, the plated cells were pre-treated with the highest non-cytotoxic concentrations of compounds for 2 h and then stimulated with 1 µg/mL of LPS (lipopolysaccharide from *Escherichia coli* 0111:B4; Sigma Aldrich) for an additional 22 h. After the incubation period, cell culture supernatants of each group were collected and mixed with the same volumetric amount of Griess reagent (1% sulfanilamide and 0.1% N-(1- naphthyl) ethylenediamine dihydrochloride in 5% phosphoric acid; Fluka) for 10 min in the dark at room temperature. The optical density of the yielded chromophore is measured by using a microplate reader (Thermo Multiskan) at 540 nm wavelengths. The nitrite levels were determined by using a sodium nitrite standard calibration curve. As positive controls, indomethacin (100 µM) and an inhibitor of nitric oxide synthase L-NAME (L-N^G-Nitro arginine methyl ester; Sigma-Aldrich) (100 µM) were used.

IL-6 level

Anti-inflammatory potentials of compounds were also through the secretion of pro-inflammatory cytokine IL-6 level in LPS-activated RAW264.7 cells. For this purpose, IL-6 levels from cell supernatants of nitrite assay were determined via commercial IL-6 rat ELISA kit **(**BMS625; Invitrogen**)** according to the manufacturer's protocol in duplicates. The results were detected at 450 nm spectrophotometrically (Multiskan Ascent; ThermoFischer) and expressed as pg/mL as previously described (Erdoğan et al. 2021).

Analgesic Activity

Analgesic activity compounds were assessed by the inhibition potential of PGE_2 level in LPS-activated murine macrophage cells. The release of PGE_2 was assessed from cell supernatants by a commercial ELISA kit (ab287802; Abcam) according to the manufacturer's protocol in duplicates. The results were measured at 450 nm spectrophotometrically (Multiskan Ascent; ThermoFischer) and expressed as pg/mL (Buran et al. 2021).

Table S1. Relative cell viability, nitrite level, nitrite inhibition, and PGE_2 levels of isolated compounds from *Gentiana cruciata* in LPS-activated RAW264.7 cells.

Compound	Cell viability%	Nitrite level (µM)	Nitrite inhibition%	PGE_2 level (pg/mL)
Ctrl	102.9± 0.98	0.5± 1.72	-	49.4± 0.02
Ctrl+LPS	100.0± 2.24	39.0± 5.76	-	148.3± 2.04
1	91.5± 0.47	32.4± 2.55	16.6± 1.51	ni
2	92.7± 3.47	37.9± 2.51	3.7± 0.41	ni
3	97.2± 2.93	32.9± 3.62	15.5± 4.77	ni
4	98.7± 0.64	28.8± 7.52	25.8± 3.22	100.2± 16.79 [a]
5	102.3± 2.46	41.6± 5.62	ni	ni
6	89.8± 4.90	30.7± 2.91	18.1± 4.29	ni
7	95.0± 6.48	30.1± 2.12	22.9± 0.44	ni
8	92.8± 3.27	36.9± 5.92	5.6± 1.71	ni
9	94.3±1.15	22.7± 5.37	39.4± 7.68	ni
10	95.8± 6.08	35.1± 5.16	10.4± 2.67	ni
11	93.6± 2.05	36.5± 4.56	5.9± 1.70	ni
L-NAME	89.3± 1.35	29.6± 6.54	25.1± 5.12	ni
IND	87.7± 5.28	28.2± 3.17	26.7± 4.19	94.4± 13.98 [b]

Ctrl: Control group treated with culture medium; Ctrl+LPS: Control group activated with LPS; IND: Indomethacin (100 µM), ni: No inhibition; L-NAME: N^G-nitro-L-arginine methyl ester hydrochloride (100 µM); LPS: Lipopolysaccharide from *Escherichia coli* 0111:B4. All isolated compounds were tested at 200 µM. The results were expressed as mean± SD. The significant differences between groups and Ctrl+LPS were defined with [a]$p<0.05$; [b]$p<0.01$.

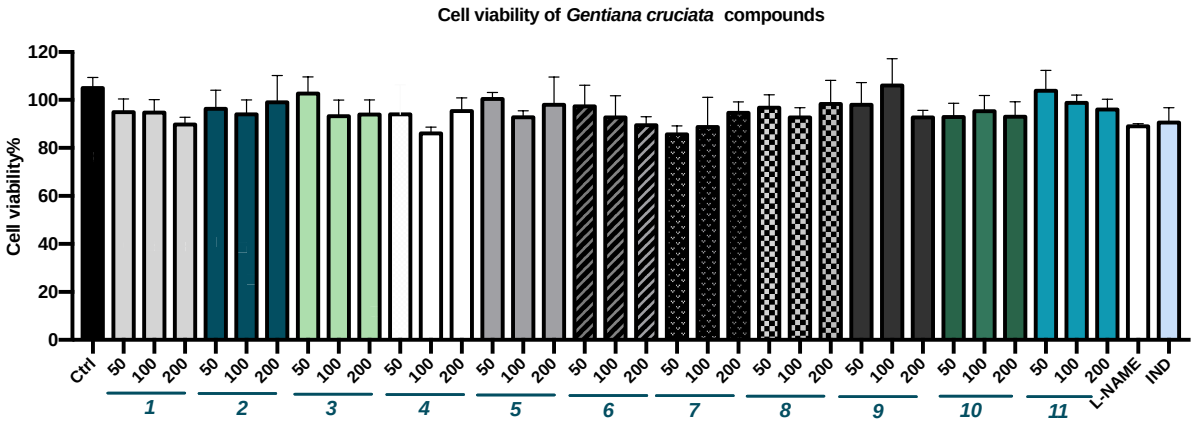

Figure S2. Cytotoxicity profile of compounds isolated from *Gentiana cruciata* in RAW264.7 cells for 24 h exposure.

All concentrations were indicated in µM unit. IND: Indomethacin (100 µM),; L-NAME: NG-nitro-L-arginine methyl ester hydrochloride (100 µM). The results were expressed as mean± SD.

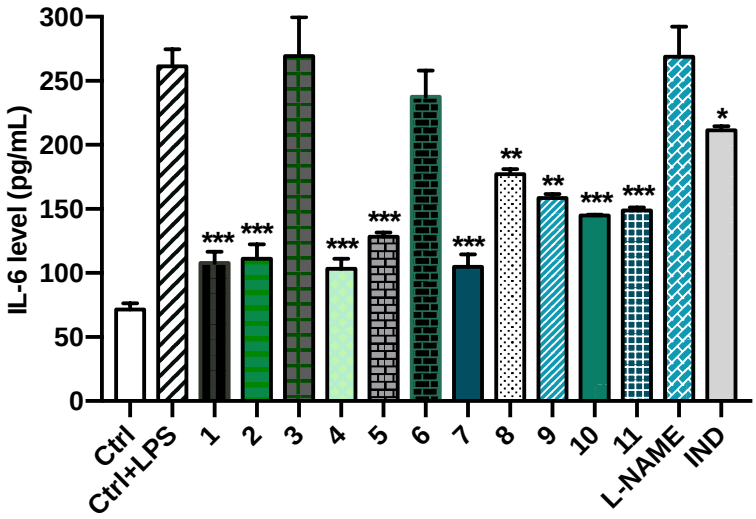

Figure S3. IL-6 secretion of LPS-activated RAW264.7 cells pre-treated with compounds.
Ctrl: Control group treated with culture medium; Ctrl+LPS: Control group activated with LPS; IND: Indomethacin (100 µM); L-NAME: N^G-nitro-L-arginine methyl ester hydrochloride (100 µM); LPS: Lipopolysaccharide from *Escherichia coli* 0111:B4. All isolated compounds were tested at 200 µM. The results were expressed as mean± SD. The significant differences between groups and Ctrl+LPS were defined with $^{*}p<0.05$ $^{**}p<0.01$ and $^{***}p<0.001$.

References

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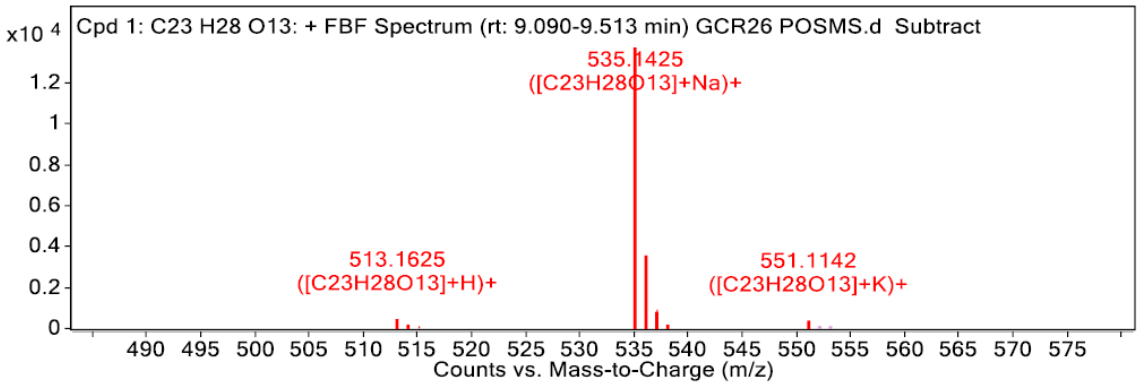


Figure S4. HRESIMS spectrum of compound **1**.

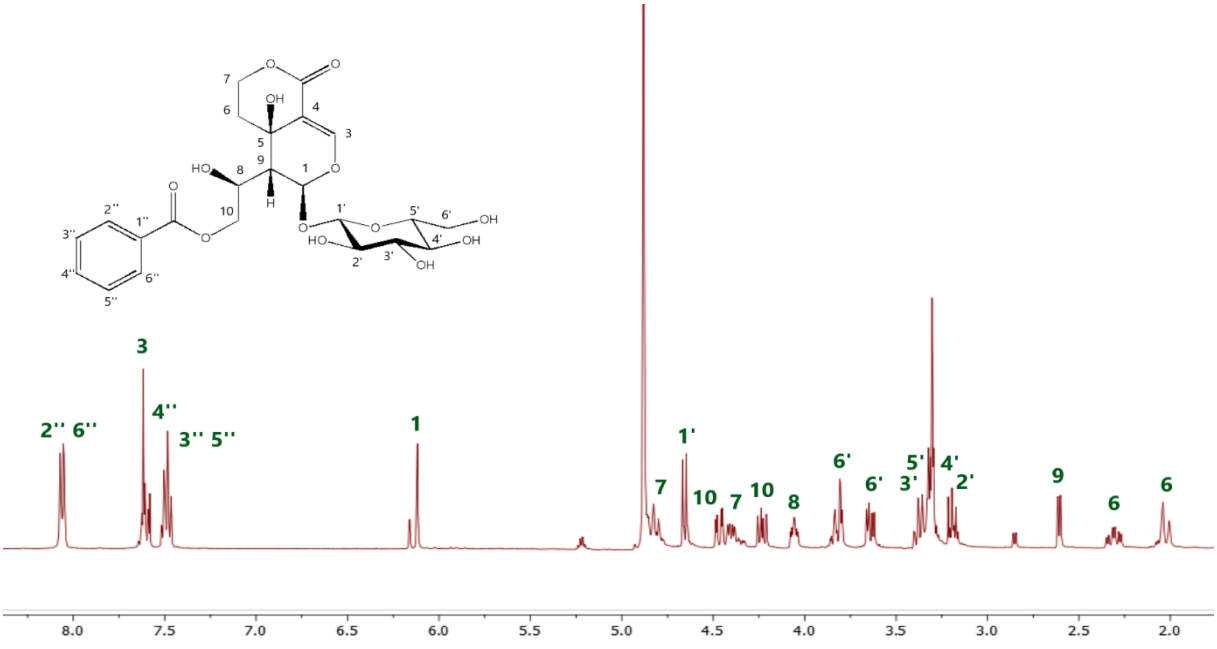


Figure S5. ^{1}H NMR spectrum of compound **1**.

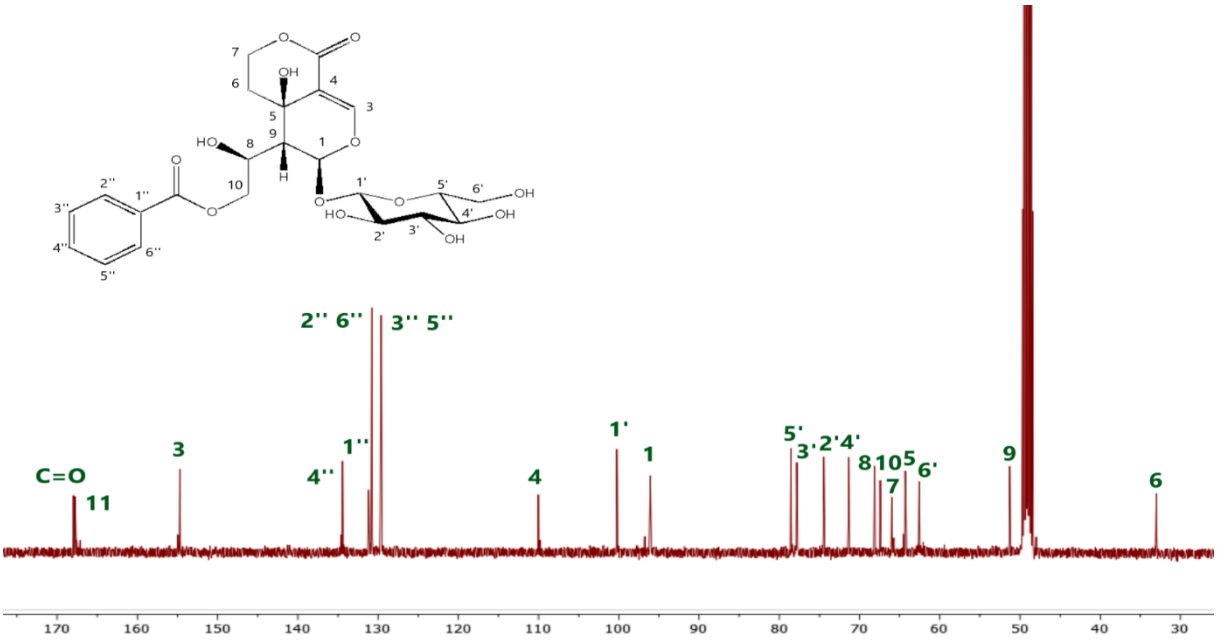


Figure S6. JMOD ^{13}CNMR spectrum of compound **1**.

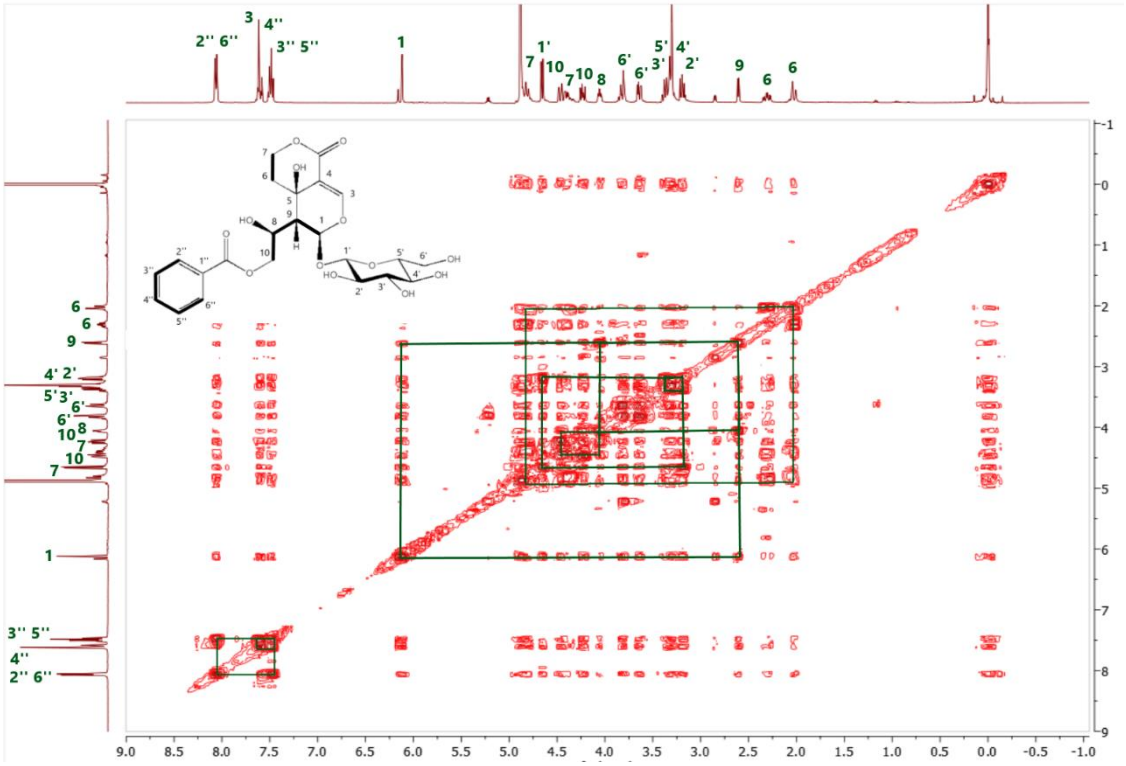


Figure S7. COSY spectrum of compound **1**.

Figure S8. HSQC spectrum of compound **1**.

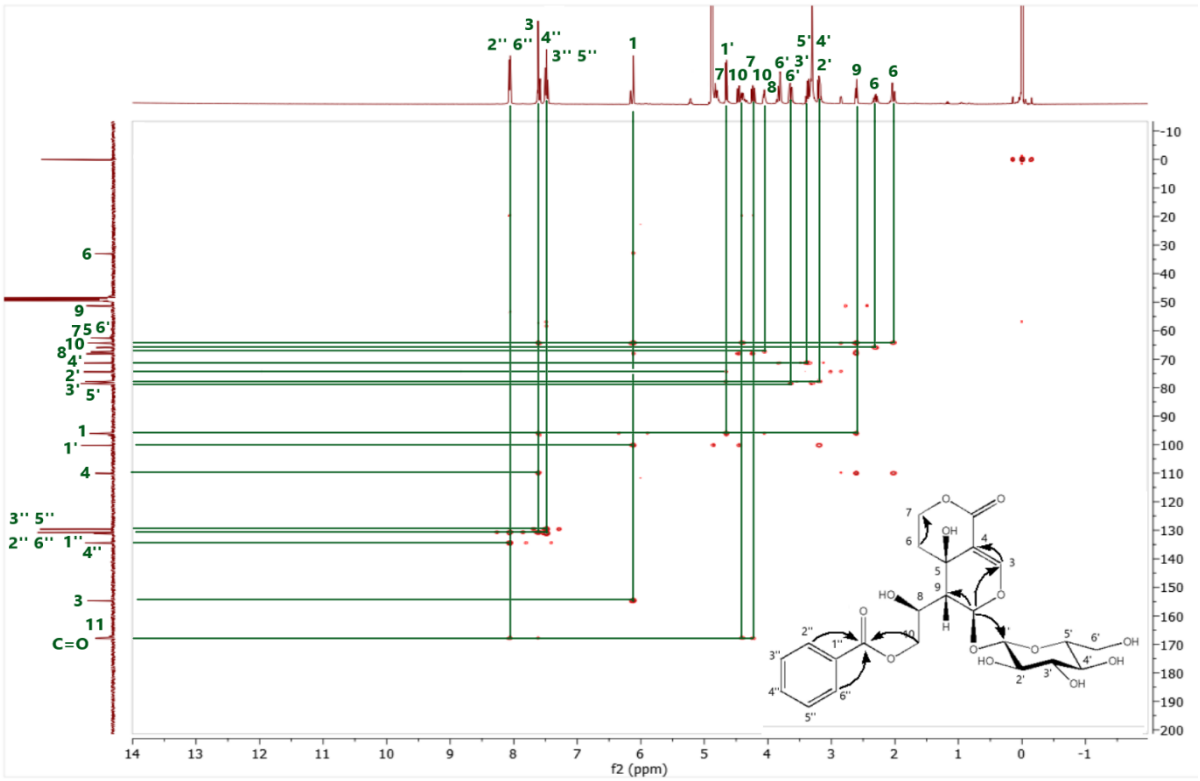


Figure S9. HMBC spectrum of compound **1**.

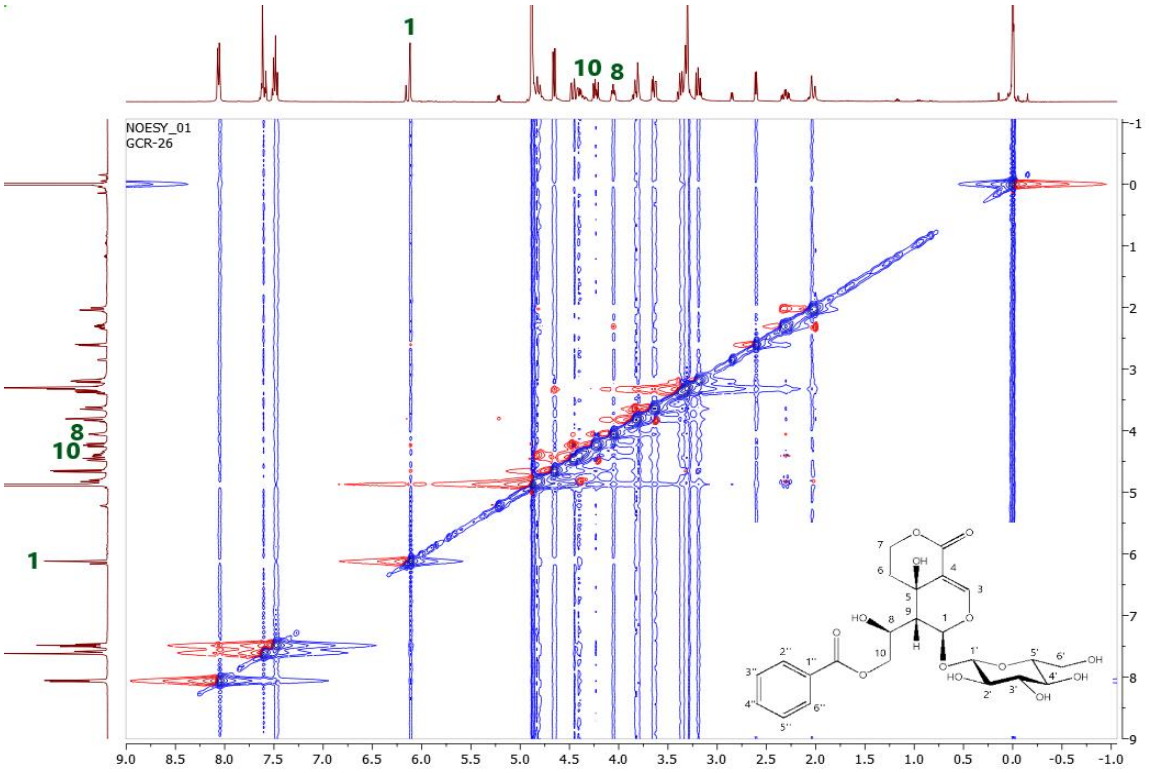


Figure S10. NOESY spectrum of compound **1**.

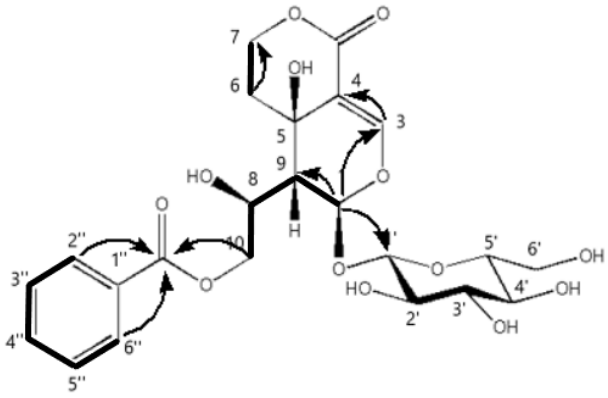
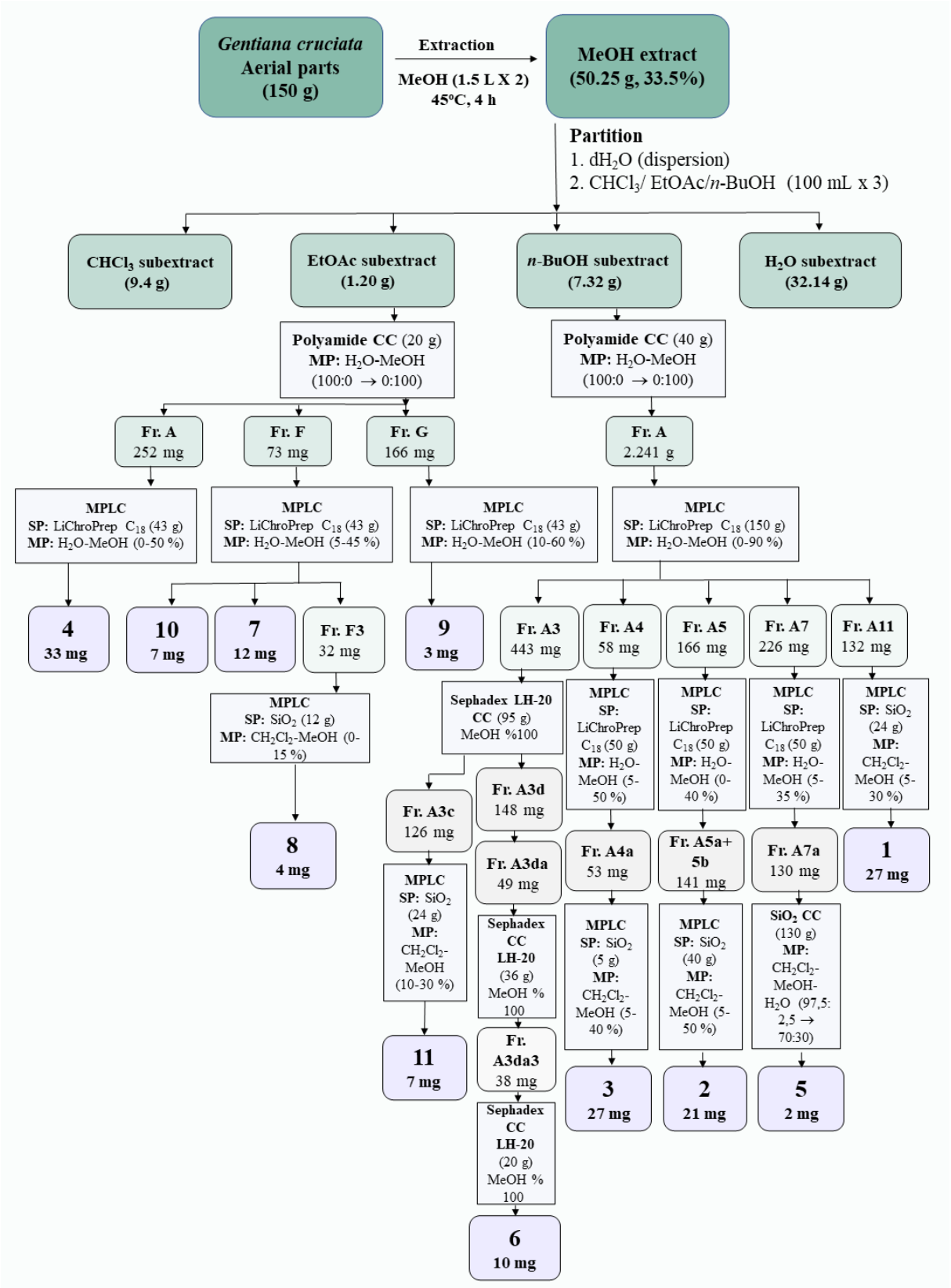


Figure S11. Key HMBC (arrow, H→C) and ^{1}H-^{1}H-COSY (bold) correlations of **1**.

Table S2. The NMR data for the isolated compounds (**1** and **3)** and eustomorusside in CD_3OD.

	1		**3**		Eustomorusside[a]	
Position	δ_C(ppm)	δ_H(ppm), *J* (Hz)	δ_C(ppm)	δ_H(ppm), *J* (Hz)	δ_C(ppm)	δ_H(ppm), *J* (Hz)
Aglycone						
1	96.0	6.12 d (1.2)	96.4	6.00 d (1.2)	98.5	6.01 br s
3	154.7	7.62 s	154.7	7.57 s	154.6	7.60 s
4	110.0	-	109.9	-	110.1	-
5	64.4	-	64.7	-	64.7	-
6	32.9	2.31 m 2.02 br.d (13.0)	33.2	2.16 m / 2.01 br.d (13.8)	33.3	2.16 br dd (13, 5) 2.00 br d (13)
7	65.8	4.81 m 4.40 m	65.9	4.80 m 4.37 m	65.9	4.80 br dd (12, 11) 4.62 d (8)
8	68.1	4.06 m	70.8	3.62 m	70.8	3.60-3.30 m
9	51.2	2.61 dd (5.7, 1.2)	50.9	2.48 dd (6.2,1.1)	50.4	2.48 d (7.0)
10	67.4	4.47 dd (11.8, 2.9) 4.23 dd (11.8, 7.4)	64.7	3.61 dd (6.2, 3.2) 3.55 m	64.7	3.60-3.30
11	167.7	-	168.0	-	168.0	-
Glucose						
1′	100.2	4.65 d (7.9)	100.0	4.62 d (7.9)	100.0	4.62 d (8)
2′	74.4	3.19 dd (9.0, 7.9)	74.4	3.17 dd (9.0, 7.9)	74.5	
3′	77.8	3.38 t (9.0)	77.8	3.37 t (9.0)	77.9	
4′	71.3	3.31 *	71.4	3.29 *	71.5	
5′	78.5	3.32 *	78.5	3.33 *	78.5	
6′	62.3	3.82 dd (12.0, 1.6) 3.64 dd (12.0, 5.2)	62.6	3.89 dd (12.0, 2.0) 3.67 dd (12.0, 5.6)	62.7	3.89 dd (12, 1) 3.60 dd (12, 5)
Benzoyl						
1″	131.1	-				
2″	130.8	8.06 dd (7.8, 1.3)				
3″	129.6	7.48 t (7.8)				
4″	134.4	7.61 m				
5″	129.6	7.48 t (7.8)				
6″	130.8	8.06 dd (7.8, 1.3)				
CO	167.9	-				

* Signal pattern unclear due to overlapping. [a] Data from Mpondo et al. 1990.



Schema S1. Extraction and isolation of compounds (**1-11**) from *G. cruciata.*