

# Iridoids and flavonoids from the aerial parts of *Gentiana asclepiadea* L. with anti-inflammatory and analgesic activities

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**ABSTRACT:** Using various chromatographic methods, seven compounds, including two secoiridoid glycosides; depressine (1) and gentiopicroside (2), one iridoid glycoside; loganic acid (3), two flavone-C-glycosides; isoorientin (4) and isovitexin (5), one xanthone-C-glycoside, mangiferin (6) as well as a nucleoside; adenosine (7) were isolated from the MeOH extract prepared from the aerial parts of *Gentiana asclepiadea* L. Their structures were elucidated unambiguously by spectroscopic methods such as 1D and 2D NMR as well as HRESIMS. The anti-inflammatory and analgesic activities of the isolated compounds were also evaluated *in vitro*. Among the tested compounds, 1, 2, and 4 showed potent anti-inflammatory activity through both nitrite and IL-6 pathways at 200 µM. Besides, compound 1 exhibited the highest decrease in PGE<sub>2</sub> level, with a higher inhibition rate compared to positive control indomethacin.

**KEYWORDS:** *Gentiana asclepiadea*; Gentianaceae; secoiridoid; depressine; anti-inflammatory and analgesic activity

## 1. INTRODUCTION

The genus *Gentiana* consists of 400 species widely distributed all over the world, mainly in Asia and Europe [1]. Several members of the genus have been used in traditional medicine for the treatment of numerous ailments. Qin-Jiao, a well-known Chinese folk remedy prepared from the roots of *Gentiana macrophylla*, *G. crassicaulis*, *G. straminea*, and *G. dahurica* has been utilized to treat rheumatoid arthritis, stroke, pains, and jaundice [2] whereas the whole plant of *G. veitchiorum* has been consumed against liver jaundice, chronic pharyngitis, and headache in Traditional Tibetan Medicine [3]. *G. lutea* exhibits diuretic, stomachic, anti-inflammatory, and wound-healing activity, also listed as *Gentianae radix*, an official drug in many pharmacopeias [4,5]. There are 12 species of *Gentiana* growing wild in the flora of Türkiye [6]. Among these species, *G. asclepiadea* L. has been used to stimulate appetite and against fever in Anatolian traditional medicine [7]. Roots and rhizomes of this species have been employed as folk remedies against gall and liver diseases, flatulence, diarrhea, and loss of appetite in the Balkan Peninsula [8–11].

Recent pharmacological studies on *G. asclepiadea* extracts demonstrated a wide range of bioactivities including antioxidant, antimicrobial, antigenotoxic, and hepatoprotective effects [12–15]. Few previous phytochemical investigations on this species indicated the presence of secoiridoids, flavonoids, and xanthone derivatives, [16–19]. However, no detailed study has been conducted on the isolation of its secondary metabolites and their *in vitro* anti-inflammatory and analgesic activities.

The inflammatory response is a defense mechanism that comprises both local and systemic reactions against various agents to support tissue regeneration and the healing process. Pro- and anti-inflammatory mediators regulate inflammatory reactions [20] and restore body homeostasis. These mediators include cytokines, prostaglandins, chemokines, vasoactive peptides, and amines [21]. Nitric oxide (NO) is a free radical synthesized from L-arginine and regulates vascular tone [22] whereas pro-inflammatory cytokine interleukin-

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6 (IL-6) affects antigen-specific immune responses, hematopoiesis, and apoptosis [23]. Moreover, prostaglandins are key mediators in the generation of inflammatory edema and pain response [24].

As a part of our ongoing efforts on the isolation of bioactive secondary metabolites from Turkish *Gentiana* species [25,26], we attempted to isolate the secondary metabolites from the aerial parts of *G. asclepiadea*. We report here the isolation, structure elucidation as well as *in vitro* anti-inflammatory and analgesic potentials of seven compounds obtained from *G. asclepiadea*.

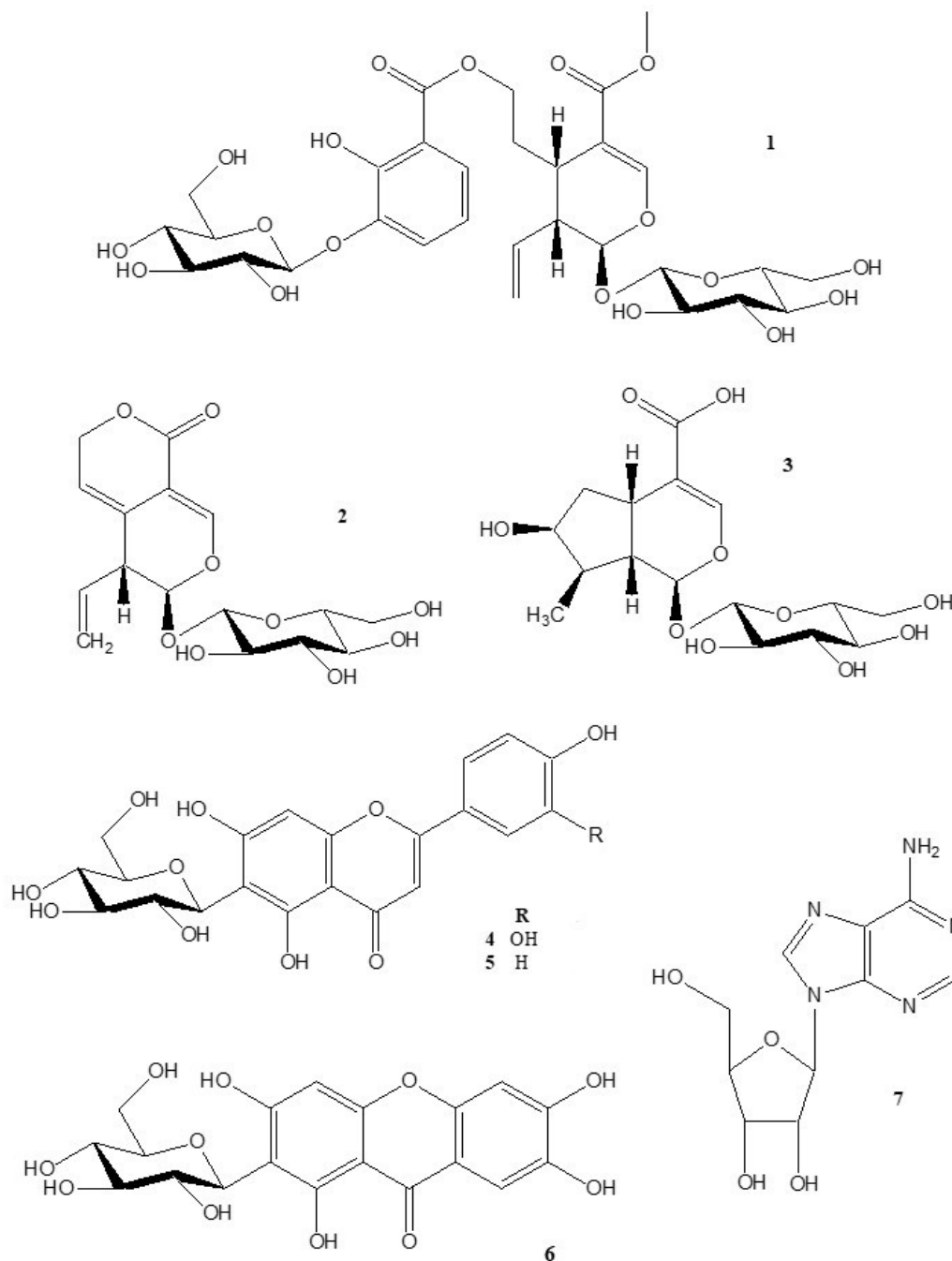


Figure 1. Chemical structures of compounds 1-7 isolated from *G. asclepiadea*.

## 2. RESULTS AND DISCUSSION

### 2.1. Structure elucidation of the isolates

The shade-dried and powdered aerial parts of *G. asclepiadea* were extracted with MeOH. The crude MeOH extract was then dispersed in H<sub>2</sub>O and partitioned with CHCl<sub>3</sub>, EtOAc, and *n*-BuOH, respectively. As a result of consecutive chromatographic methods, seven compounds (1-7) (Figure 1) were purified from the EtOAc and *n*-BuOH subextracts. The structures were identified as depressine (1) [27], gentiopicroside (2) [28],

loganic acid (3) [29], isoorientin (4) [30], isovitexin (5) [31], mangiferin (6) [32] and adenosine (7) [33] by comparison of their spectroscopic data with those reported in the literature. In previous studies, compounds 2-6 were detected in *G. asclepiadea* extracts by HPLC analysis, while the presence of 1 was tentatively identified [34-36]. In our very recent study, compounds 2-6 were also reported from another *Gentiana* species, *G. cruciata* [25]. However, only a few of them were isolated from the title species, *G. asclepiadea* [28,37]. Depressine (1) and loganic acid (3) were isolated from different *Gentiana* species in previous studies [27,38]. However, our study is the first record of the isolation of compounds 1 and 3 from *G. asclepiadea*. Depressine (1) is a unique and rare secoiridoid glycoside bearing a phenolic subunit like oleuropein. Its exact structure was elucidated based on 1D and 2D-NMR spectra (Figures 2-5) as well as MS analysis. To the best of our knowledge, it is being reported for the third time from a natural source after *Gentiana depressa* and *G. szechenyii* [27,39]. Moreover, its pharmacological activity has never been studied before.

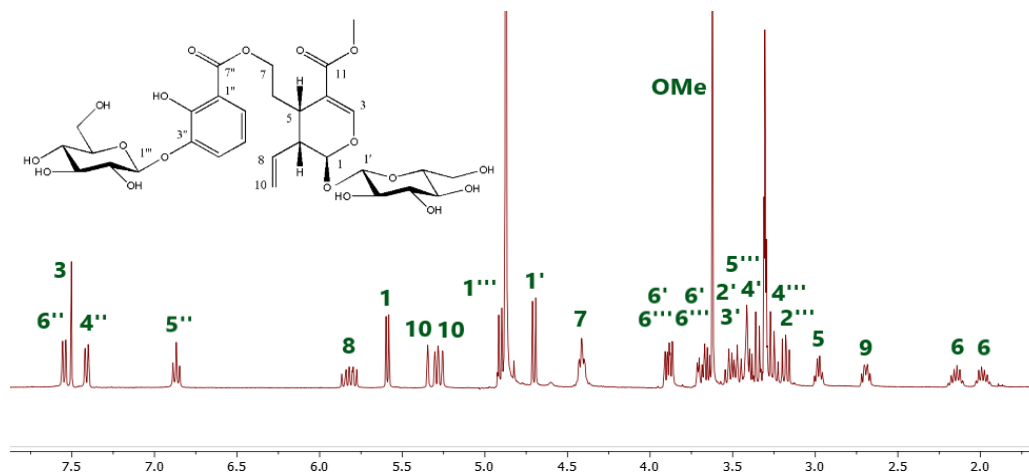


Figure 2.  $^1\text{H-NMR}$  Spectrum (500 MHz,  $\text{CD}_3\text{OD}$ ) of 1.

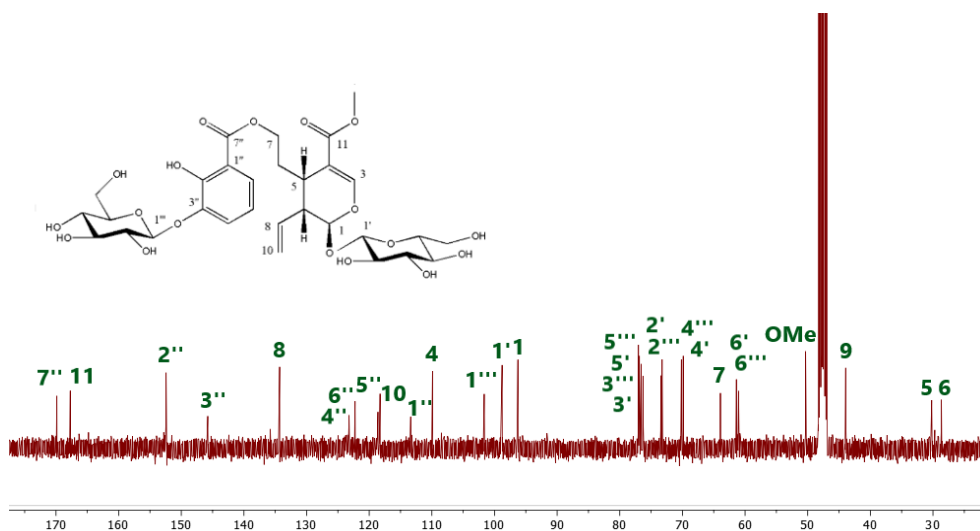


Figure 3.  $^{13}\text{C-NMR}$  Spectrum (100 MHz,  $\text{CD}_3\text{OD}$ ) of 1.

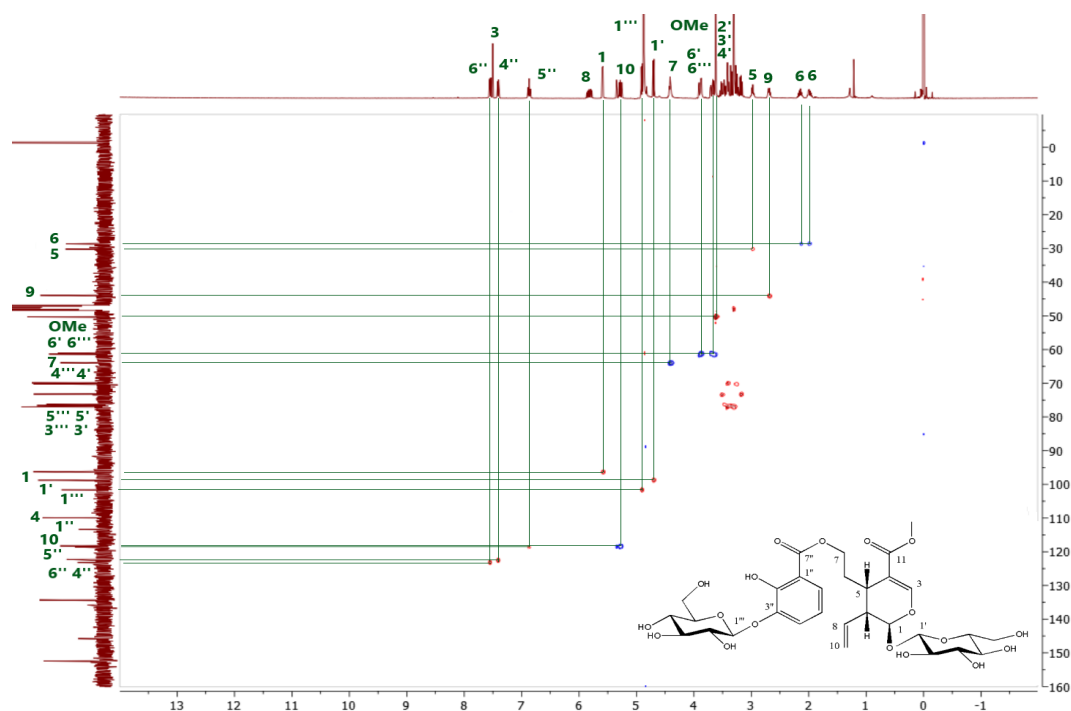


Figure 4. HSQC Spectrum of 1.

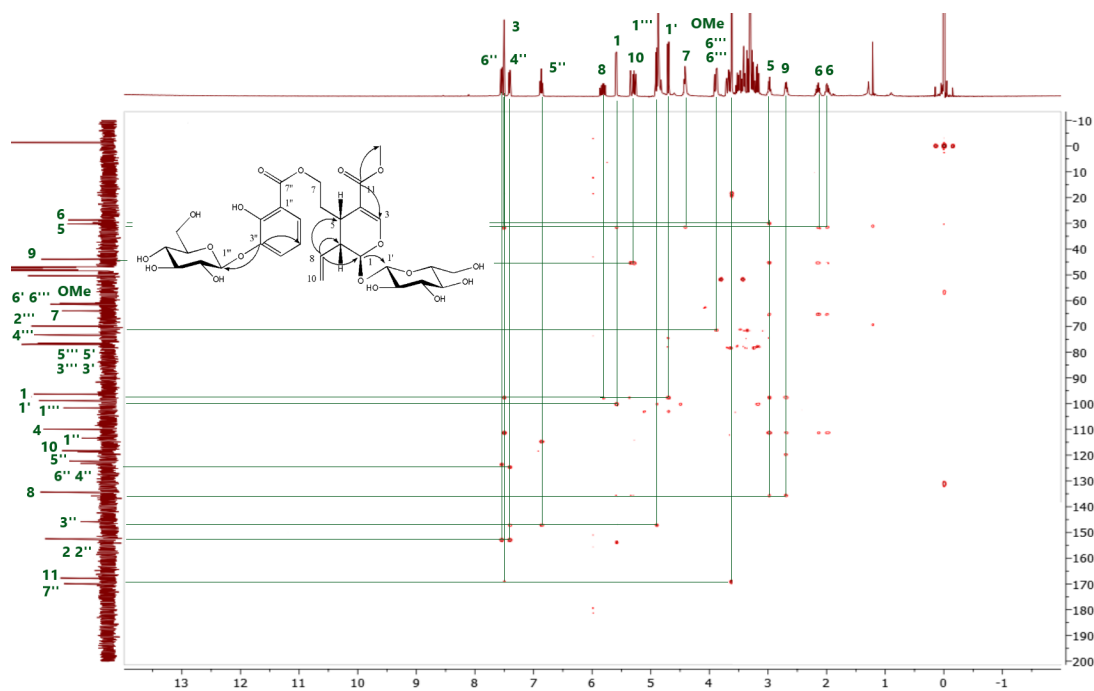
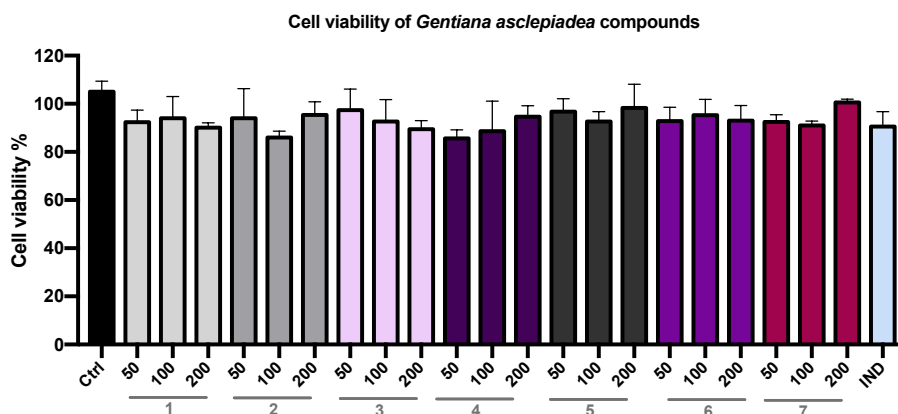


Figure 5. HMBC Spectrum of 1.

## 2.2. Cytotoxicity and MTT assay

RAW 264.7 cells were treated with different concentrations (25-200  $\mu\text{M}$ ) of compounds for 24 h of exposure by MTT assay. According to the statistical analysis, no significant difference was observed between tested doses. Although a slight reduction was observed in the mean cell viability at 100  $\mu\text{M}$  for **compounds 2 and 5**, there was no significant difference between the tested doses. In addition, the results revealed that none of the compounds caused remarkable cytotoxicity at the highest tested concentration, 200  $\mu\text{M}$ , with relative cell viabilities above 70% compared to the control group (Figure 6, Table 1). Thus, 200  $\mu\text{M}$  was used for further anti-inflammatory and analgesic studies.

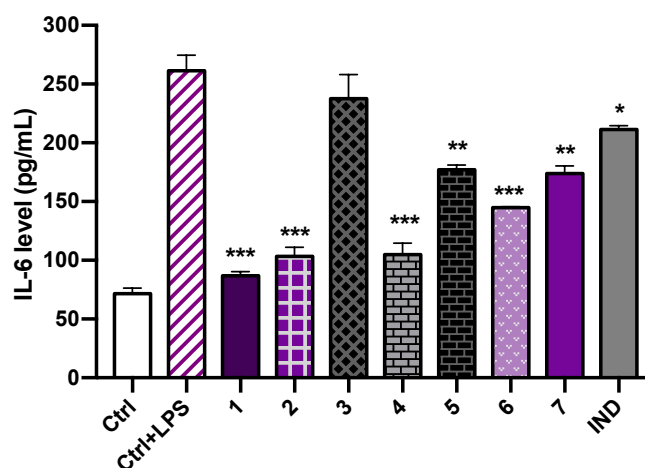


**Figure 6.** Cytotoxicity of compounds 1-7 on RAW264.7 cells for 24 h exposure. All concentrations were indicated in μM. IND: Indomethacin (100 μM). The results were expressed as mean± SD.

### 2.3. Anti-inflammatory activity

Nitric oxide (NO) plays a key role in the regulation of immunity and inflammation. It is generated in response to pro-inflammatory and mitogenic stimuli, including bacterial lipopolysaccharide (LPS) [40]. Nitrite (NO<sub>2</sub>) is a stable and nonvolatile breakdown product of NO and also a biomarker of NO metabolism [41]. Since NO is difficult to quantify due to its short half-life in the presence of O<sub>2</sub> and other scavenging radicals, stable degradation products (such as NO<sub>2</sub>) are used to indicate the presence of NO indirectly. In our study, the amount of nitrite was determined by a spectrophotometric assay based on the reaction of NO<sub>2</sub> with the Griess reagent [42]. On the other hand, pro-inflammatory cytokine IL-6 is generated in response to tissue damage and infections [43]. LPS stimulation of macrophages was used in order to simulate *in vitro* induction of pro-inflammatory cytokine, IL-6 [44]. In our study, the anti-inflammatory activity of the isolates was assessed by a commercial IL-6 ELISA kit in LPS-activated RAW 264.7 cells.

As shown in Table 1 and Figure 7, LPS significantly induced both nitrite level and IL-6 release in RAW 264.7 cells. Pre-treatment with all isolated compounds led to nitrite inhibition, most potently with compounds 1, 2, and 4, with a relative nitrite inhibition of over 20% compared to the control. Besides, pro-inflammatory IL-6 release displayed a different profile in the RAW 264.7 cells pre-treated with the isolated compounds. According to the results, depressine (1) exerted the highest decrease in IL-6 level among all tested compounds (p<0.001) (Figure 7). These results indicated that compounds 1, 2, and 4 showed a potent anti-inflammatory activity by inhibiting the levels of both nitrite and IL-6 in LPS-activated RAW 264.7 cells.



**Figure 7.** IL-6 secretion of LPS-activated RAW264.7 cells pre-treated with compounds 1-7.

Ctrl: Control group treated with culture medium; Ctrl+LPS: Control group activated with LPS; IND: Indomethacin (100 μM); LPS: Lipopolysaccharide from *Escherichia coli* 0111:B4. All 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.

## 2.4. Analgesic activity

Prostaglandins are lipid mediators that are involved in the mediation of inflammatory pain. Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) has long been investigated for developing novel therapeutic strategies for the treatment of pain [45]. In our study, the analgesic activities of compounds 1-7 were evaluated by using a commercial ELISA kit in LPS-activated RAW 264.7 cells. Among the tested compounds, depressine (1) led to the highest decrease in PGE<sub>2</sub> level, which has a notable inhibition rate compared to the positive control, indomethacin (Table 1).

Several studies have been conducted to evaluate the anti-inflammatory and analgesic activities of gentiopicoside (2) [46–48]. Previous *in vitro* experiment results indicated that gentiopicoside (2) inhibited NO, IL-6, and PGE<sub>2</sub> in LPS-activated RAW 264.7 cells [25,49]. Loganic acid (3), isoorientin (4), and isovitexin (5) were also reported to possess anti-inflammatory activity [25,50–52]. Loganic acid (3) exhibited *in vivo* anti-inflammatory effect by reducing TNF- $\alpha$  and IL-6 activity [53]. The anti-inflammatory activity of isoorientin was evaluated through both *in vivo* and *in vitro* assays such as expression of COX-2, iNOS, 5-LOX, TNF- $\alpha$ , IL1- $\beta$ , activation of NF- $\kappa$ B, and also paw edema, and air pouch models.

**Table 1.** Effects of compounds (1-7) on cell viability, nitrite level, nitrite inhibition and PGE<sub>2</sub> levels in LPS-activated RAW264.7 cells.

Ctrl: Control group treated with culture medium; Ctrl+LPS: Control group activated with LPS; IND: Indomethacin (100  $\mu$ M), ni: No inhibition; LPS: Lipopolysaccharide from *Escherichia coli* 0111:B4. All isolated compounds were tested at 200  $\mu$ M. The results were expressed as mean  $\pm$  SD. The significant differences between groups and Ctrl+LPS were defined with \* $p$ <0.05 \*\* $p$ <0.01. <sup>a</sup>Results are from our very recent study [25].

Compound	Cell viability%	Nitrite level ( $\mu$ M)	Nitrite inhibition%	PGE <sub>2</sub> level (pg/mL)
Ctrl	102.98 $\pm$ 0.98	0.49 $\pm$ 1.72	-	49.43 $\pm$ 0.02
Ctrl+LPS	100.00 $\pm$ 2.24	39.00 $\pm$ 5.76	-	148.34 $\pm$ 2.04
1	89.58 $\pm$ 1.73	28.72 $\pm$ 7.36	23.97 $\pm$ 3.36	47.28 $\pm$ 0.51**
2 <sup>a</sup>	98.74 $\pm$ 0.64	28.86 $\pm$ 7.52	25.84 $\pm$ 3.22	100.18 $\pm$ 16.8*
3 <sup>a</sup>	89.86 $\pm$ 4.90	30.71 $\pm$ 2.91	18.11 $\pm$ 4.29	ni
4 <sup>a</sup>	95.01 $\pm$ 6.48	30.11 $\pm$ 2.12	22.90 $\pm$ 0.44	ni
5 <sup>a</sup>	92.81 $\pm$ 3.27	36.92 $\pm$ 5.92	5.68 $\pm$ 1.71	ni
6 <sup>a</sup>	95.82 $\pm$ 6.08	35.14 $\pm$ 5.16	10.44 $\pm$ 2.67	ni
7	100.58 $\pm$ 1.93	29.88 $\pm$ 3.24	18.93 $\pm$ 5.22	ni
IND	87.72 $\pm$ 5.28	28.18 $\pm$ 3.17	26.77 $\pm$ 4.19	94.45 $\pm$ 13.98**

Results confirmed the anti-inflammatory effect of isoorientin treatment in those LPS-induced RAW 264.7 cell lines and carrageenan-induced inflammatory animal model systems [54]. Isovitexin displayed anti-inflammatory effects through TNF- $\alpha$ , IL-6, iNOS, and COX-2 levels and MAPK and NF- $\kappa$ B signaling pathways in LPS-induced RAW 264.7 cells [55]. Moreover, mangiferin (6) showed dose-dependently inhibition of LPS-induced NO and PGE<sub>2</sub> secretions in RAW 264.7 macrophages and peritoneal macrophages isolated from C57BL/6 mice [56]. To our best knowledge, this is the first report concerning the biological activity of depressine (1), which deserves further *in vitro* and *in vivo* studies.

## 3. CONCLUSION

The phytochemical investigations on the EtOAc and *n*-BuOH subextracts of the crude MeOH extract from *G. asclepiadea* yielded depressine (1), gentiopicoside (2), loganic acid (3), isoorientin (4), isovitexin (5), mangiferin (6) and adenosine (7). Their structures were elucidated on the basis of spectroscopic analysis. Depressine (1) and loganic acid (3) were isolated from *G. asclepiadea* for the first time. Amongst the tested isolates, particularly depressine (1) exhibited promising anti-inflammatory and analgesic activities via decreasing the levels of NO, IL-6 and PGE<sub>2</sub> in LPS-induced RAW 264.7 macrophage cell lines. Thus, depressine (1) deserves further *in vitro* and *in vivo* anti-inflammatory and analgesic activity studies on the way to discover new anti-inflammatory and analgesic drug leads.



## 4. MATERIALS AND METHODS

### 4.1. General experimental procedures

Silica gel 60 (Merck, Germany), Sephadex LH-20 (Sigma-Aldrich, USA) and Polyamide (Fluka Analytical, Sigma-Aldrich, USA) were used for Column chromatography (CC). For medium-pressure liquid chromatographic (MPLC) separations, the SepacoreVR Flash Systems X10/X50 (Buchi Labortechnik AG, Switzerland) was utilized with RediSep columns (LiChroprep C<sub>18</sub> and SiO<sub>2</sub>; Teledyne Isco, USA). TLC analyses were carried out on silica gel 60 F<sub>254</sub> precoated plates (Merck, Germany), visualization was performed by spraying with 1% vanillin in concentrated H<sub>2</sub>SO<sub>4</sub> solution followed by heating at 105 °C for 2-3 minutes and detected with UV lights at 254 and 365 nm. The solvents used for chromatographic separations were of analytical grade. UV Spectra were recorded by using HP Agilent 8453 spectrophotometer (Agilent Technologies, USA, λ<sub>max</sub> in nm). IR Spectra (KBr) were recorded by using PerkinElmer 2000 FT-IR spectrometer (PerkinElmer, USA, ν in cm<sup>-1</sup>), respectively. 1D (<sup>1</sup>H: 500 and 400 MHz, <sup>13</sup>C: 125 and 100 MHz) and 2D (COSY, HSQC, HMBC, and NOESY) NMR experiments were performed on a Bruker Avance DRX 500 spectrometer (Bruker, USA) in deuterated methanol (CD<sub>3</sub>OD) and solvent signals were taken as references. The chemical shift values (δ) were presented in ppm and coupling constants (J) are in Hz. HRESIMS data were recorded on a Thermo Scientific QExactive Plus Orbitrap Mass spectrometer in positive ionization mode. ESI-MS was recorded on Agilent 6200 series TOF/6500 series mass spectrometer (Agilent Technologies, USA).

### 4.2. Plant material

The above-ground parts of *Gentiana asclepiadea* L. were collected from Devrekani, Kastamonu province of Türkiye in August 2019. It was identified by Prof. Dr. Hasan Kirmızibekmez and a voucher specimen (YEF 19042) was kept in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Yeditepe University, İstanbul, Türkiye.

### 4.3. Extraction and isolation

The shade-dried and powdered aerial parts of *G. asclepiadea* were extracted twice with MeOH (1.5 L) at 45 °C for 4 h. The pooled extracts were dried under vacuum to yield a residue (56 g, yield 37 %). The crude MeOH extract was dispersed in distilled H<sub>2</sub>O (100 mL) and submitted to liquid-liquid extraction in a separatory funnel with equal volumes of CHCl<sub>3</sub> (3 x 100 mL), EtOAc (3 x 100 mL), and *n*-BuOH (3 x 100 mL) to obtain the subextracts of CHCl<sub>3</sub> (13.33 g), EtOAc (1.65 g), *n*-BuOH (12.21 g), and H<sub>2</sub>O (23.49 g). EtOAc subextract was loaded to the polyamide column (20 g) eluting with H<sub>2</sub>O (200 mL) and a stepwise gradient of MeOH in H<sub>2</sub>O (0→100% in steps of 10%, each 100 mL) to yield ten fractions, frs. 1-10. Fr. 2 (663 mg) was subjected to SiO<sub>2</sub> (60 g) column eluting with CH<sub>2</sub>Cl<sub>2</sub>-MeOH (100:0 to 50:50) to give 2 (27 mg). *n*-Butanol subextract was fractioned over polyamide column (70 g) eluting with H<sub>2</sub>O (200 mL) and a stepwise gradient of MeOH in H<sub>2</sub>O (0→100% in steps of 10%, each 100 mL) to yield nine fractions, frs. A-I. Fr. C (3 g) was applied to SiO<sub>2</sub> (150 g) CC eluting with CH<sub>2</sub>Cl<sub>2</sub>-MeOH (100:0 to 55:45) to obtain 12 fractions, frs. C<sub>1-12</sub>. Fr. C<sub>11</sub> (173 mg) was subjected to C<sub>18</sub>-MPLC (43 g) by using H<sub>2</sub>O-MeOH (10 to 55% MeOH) to afford five subfractions, frs. C<sub>11a-11e</sub>. Purification of 1 (10 mg) was carried out from fr. C<sub>11e</sub> (21 mg) by using SiO<sub>2</sub> (5 g) CC eluting with CH<sub>2</sub>Cl<sub>2</sub>-MeOH (95:5 to 75:25). Fr. C<sub>12</sub> (205 mg) was subjected to C<sub>18</sub>-MPLC (43 g) by using H<sub>2</sub>O-MeOH gradient (5 to 50% MeOH) to afford five subfractions, frs. C<sub>12a-12e</sub>. Fr. C<sub>12a</sub> (108 mg) was subjected to Sephadex LH-20 column (20 g) and eluted with MeOH to obtain 3 (13 mg). Likewise, fr. C<sub>12c</sub> (17 mg) was loaded to the Sephadex LH-20 column (10 g) and eluted with MeOH to obtain 7 (2 mg). Fr. F (579 mg) was subjected to C<sub>18</sub>-MPLC (130 g) by using H<sub>2</sub>O-MeOH (0 to 45% MeOH) to afford seven fractions, frs. F<sub>1-7</sub>. Fr. F<sub>4</sub> (76 mg) was subjected to SiO<sub>2</sub> (12 g) CC eluting with CH<sub>2</sub>Cl<sub>2</sub>-MeOH-H<sub>2</sub>O (95:5:0.5 to 61:32:2) to obtain seven subfractions, frs. F<sub>4a-4g</sub>. Purification of 6 (3 mg) was accomplished from Fr. F<sub>4f</sub> (18 mg) by using Sephadex LH-20 column (6 g) eluted with MeOH. Fr. F<sub>5</sub> (50 mg) was subjected to SiO<sub>2</sub> (9 g) CC eluting with CH<sub>2</sub>Cl<sub>2</sub>-MeOH-H<sub>2</sub>O (90:10:1 to 50:50:2) to obtain five subfractions (frs. F<sub>5a-5e</sub>) and the latter (31 mg) was loaded to C<sub>18</sub>-MPLC (43 g) by using H<sub>2</sub>O-MeOH (20 to 50% MeOH) to give three subfractions, frs. F<sub>5e1-5e3</sub>. Purification of 4 (2 mg) was carried out from fr. F<sub>5e3</sub> (19 mg) by using Sephadex LH-20 column (6 g) eluted with MeOH. Fr. G (2063 mg) was subjected to SiO<sub>2</sub> (100 g) CC eluting with CH<sub>2</sub>Cl<sub>2</sub>-MeOH-H<sub>2</sub>O (95:5:0 to 60:40:4) to obtain seven fractions, frs. G<sub>1-7</sub>. Fr. G<sub>4</sub> (68 mg) was loaded to SiO<sub>2</sub> (12 g) CC eluting with CH<sub>2</sub>Cl<sub>2</sub>-MeOH (95:5 to 70:30) to purify 5 (44 mg).

### 4.4. Cytotoxicity assay

Before evaluation of the anti-inflammatory and analgesic activities of the isolates, cytotoxicity profiles of the compounds were determined by using MTT (Sigma-Aldrich, USA) colorimetric assay. The RAW 264.7 murine macrophage cells were purchased from the American Type Culture Collection (ATCC) and were

cultured in Dulbecco's Modified Eagle's Medium (DMEM; 10% FBS, v/v; Gibco, USA). The seeded cells were supplemented with 1% antibiotics (10.000 µg/mL streptomycin and 10.000 units/mL penicillin; Gibco, USA) in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C until confluency. After 24 hours, the incubated cells were treated with serial dilutions of the compounds at final concentrations between 25-200 µM. The used media was disposed of and MTT (0.5 mg/mL) was added to each well and incubated for another 2 h at 37 °C. Then, each well content was discarded, and the produced formazan crystals were dissolved by 100 µL of isopropanol. Afterward, the optical density of violet-colored chromophore was measured at 570 nm spectrophotometrically (BioTek, Germany). The viability (%) was calculated by using the following equation [57]:

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

#### 4.5. Anti-inflammatory activity

##### 4.5.1. Determination of nitrite level

Nitrite is a stable metabolite of nitric oxide (NO) and is frequently used as an inflammation indicator, which was shown to be increased during an inflammatory response. The RAW 264.7 cells were cultured at 37°C in 5% CO<sub>2</sub> in a 48-well plate for 24 h. The next day, the highest non-cytotoxic concentrations of the compounds were added and treated for 2 h, then stimulated with 1 µg/mL of LPS (lipopolysaccharide from *Escherichia coli* 0111: B4; Sigma Aldrich, USA) for an additional 22 h. After 24 h of treatment, culture supernatants of each group were collected and nitric oxide levels were estimated using Griess reagent (1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in 5% phosphoric acid; Fluka, Germany). The absorbance of the yielded chromophore was measured at a microplate reader (Thermo Multiskan, Finland) at 540 nm wavelengths whereas nitrite levels were calculated with the help of a sodium nitrite standard calibration curve. Indomethacin (100 µM; Fluka, Germany) was used as a positive control.

##### 4.5.2. Determination of IL-6 level

The anti-inflammatory activities of the isolates were evaluated through the estimation of IL-6 levels in LPS-activated RAW 264.7 cells. Therefore, IL-6 concentration from the nitrite assay cell supernatants was assessed by using a commercial IL-6 rat ELISA kit (BMS625; Invitrogen, USA), and the absorbance was measured at 450 nm spectrophotometrically (Multiskan Ascent; Thermo Fischer, Finland). Results for IL-6 levels were expressed as pg/mL and all determinations were made in duplicate according to the manufacturer's protocol.

##### 4.5.3. Analgesic activity

In order to evaluate the analgesic activity, the inhibition potential of the compounds on PGE<sub>2</sub> levels in LPS-activated murine macrophage cells was used. PGE<sub>2</sub> release of cell supernatants was assessed by a commercial ELISA kit (ab287802; Abcam, USA) and the absorbance was measured at 450 nm spectrophotometrically (Multiskan Ascent; Thermo Fischer, Finland). The levels of PGE<sub>2</sub> were expressed as pg/mL and all determinations were made in duplicate according to the manufacturer's protocol.

#### 4.6. Statistical analysis

GraphPad Prism 6.0 (La Jolla, California) was used for all statistical analyses. Data related to cell viability, anti-inflammatory activity (nitrite level), IL-6 and PGE<sub>2</sub> levels were analyzed by using one-way ANOVA following the post-hoc tests by Tukey. Differences were considered as significant p < 0.05.

**Author contributions:** Plant collection and identification: HK, Extraction, isolation and structural elucidation: RK, HK. Cell culture studies: RR, HS. Structural analysis: RK, HK, AB, JH. Writing, review, and editing: RK, HK, RR, HS, JH. All authors read and approved the manuscript.

**Conflict of interest statement:** The authors declared no conflict of interest.

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