Characterisation of four new triterpenoid saponins with nitric oxide inhibitory activity from aerial parts of Gouania leptostachya

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ABSTRACT
Four new triterpenoid saponins (gouaniasides VII–IX [1–3] and joazeiroside C [4]) and one known triterpenoid (5) were isolated from the aerial parts of Gouania leptostachya DC. (Rhamnaceae). Their structures were elucidated via one-dimensional and two-dimensional nuclear magnetic resonance spectroscopy, high-resolution electrospray ionisation–mass spectrometry, and analyses of hydrolytic cleavage results. The anti-inflammatory potential of compounds 1–3 was evaluated according to their ability to inhibit the production of nitric oxide (NO) by RAW 264.7 macrophages. All compounds at noncytotoxic concentrations significantly inhibited NO production by macrophages in a concentration-dependent manner.

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1. Introduction

Gouania leptostachya DC. (Rhamnaceae) is used as a traditional medicine in numerous countries, including Vietnam, India, and China. In Vietnam, the aerial parts of G. leptostachya are used to treat inflammation, including swelling, pain, and fall-induced injuries, and its leaves are used to assuage fever and treat poisoning and cold flu (Loi 2005; Chi 2006). Extracts of G. leptostachya exhibit various bioactive properties, such as anti-inflammatory (Dung et al. 2015), antibacterial (An 2010), antioxidant (Bamtunkh et al. 2007), and α-glucosidase inhibitory (Yao et al. 2011) properties. Some phytochemical characterisations of this plant have revealed the presence of flavonoids, such as quercetin 3-O-β-D-xylopyranosyl-(1→2)-α-L-rhamnopyranoside, quercetin 3-O-6-E-p-coumaroyl-β-D-glucopyranosyl-(1→2)-α-L-rhamnopyranoside, prodelphinidin C, prodelphinidin B3, (−)-epigallocatechin, (+)-gallocatechin (Yao et al. 2011), saponin (joazeiroside A) (Thuy et al. 2019), triterpenoids (gouanic C acid, ceanothenic acid, and gouanic A acid) (An 2010), and other compounds (benzopyrans 1-[rel 2S,3R]-3,5,7-trihydroxy-3,4-dihydro-2H-chromen-2-yl]ethenone and 1-[rel 2S,3S]-3,5,7-trihydroxy-3,4-dihydro-2H-chromen-2-yl]ethanone) (Yao et al. 2011). In this study, we isolated four new triterpenoid saponins (1–4) and one known compound (5) from the aerial parts of G. leptostachya collected from Vietnam (Figure 1) and characterised their structures. The inhibitory effects of compounds 1–3 on lipopolysaccharide-induced nitric oxide (NO) production in RAW 264.7 cells were investigated.

2. Results and discussion

Crude ethanol extract from the aerial parts of G. leptostachya DC. (Rhamnaceae) were fractionated, isolated, and purified through a combination of chromatographic
methods to obtain four new triterpenoid saponins (1–4) and one known compound, epigouanic acid A (5) (Leal et al. 2010; Nganso et al. 2020) (Figure 1). The structures of the compounds were characterised via one-dimensional and two-dimensional nuclear magnetic resonance (NMR) spectroscopy, high-resolution electrospray ionisation–mass spectrometry (HR-ESI-MS), and analyses of the derivatives of chemical reactions.

Compound 1 was obtained as a white powder. Its molecular formula (C_{49}H_{80}O_{18}) was determined via HR-ESI-MS. The spectrum exhibited a pseudo-molecular ion peak at m/z 957.5411 [M + H]^+ (calculated for C_{49}H_{81}O_{18}^+, 957.5417). The $^1$H NMR spectrum of 1 exhibited singlets at δ_{H} 0.88, 0.89, 1.06, 1.15, and 1.21 corresponding to five tertiary methyl groups; doublets at δ_{H} 1.08 (3H, J = 7.0 Hz) and 1.07 (3H, J = 6.5 Hz) corresponding to methyl groups with a geminal proton; and additional singlets at δ_{H} 4.84 and 4.87 corresponding to two exomethylene protons (Figure S1). The spectral characteristics suggested that 1 possessed an isopropyl side chain with terminal methylene. A similar structure has been observed for C_{31}-type derivatives (Schühly et al. 2000).

The $^1$H NMR spectrum of 1 also exhibited signals corresponding to protons on oxygen-bearing carbons. The signal at δ_{H} 3.22 (dd, 11.5, 4.0 Hz) corresponded to H-3α (Gossan et al. 2017). Doublets at δ_{H} 3.96 (d, 9.5 Hz) and 3.99 (overlap) corresponded to the oxymethylene protons H-18α and H-18β, respectively (Gossan et al. 2017). A comparison of the $^{13}$C NMR spectrum and distortionless enhancement by polarisation transfer spectrum of 1 with those of dammarane-type derivatives (Schühly et al. 2000; Gossan et al. 2017) revealed that 1 possessed a C_{31}-type skeleton. The signals at δ_{C} 154.1 and 108.8 indicated the presence of a side chain with terminal methylene. The signals of the carbon atoms in the A–E rings resembled those of gouaniaside VI (Gossan et al. 2017), except for the side-chain C atoms. The H-C(24) signal was absent and replaced by a quaternary C atom at δ_{C} 154.1. Moreover, a CH_{2} group was bound directly to the side chain of the gouaniaside VI skeleton, as shown by the heteronuclear multiple bond correlations (HMBCs) of H-26 (δ_{H} 1.08) and H-27 (δ_{H} 1.07) with C-24 (δ_{C} 154.1) and C-25 (δ_{C} 35.0) and H-26 with C-27 (22.1) (Figures S9 and S10). The binding was confirmed by the nuclear overhauser effect spectroscopy signals between the H atoms of CH_{2}=C(24) cis to C-23 and H-22 (Figure S12). In addition, correlations

**Figure 1.** The structures of compounds (1-5).
observed between H-3 and H-28 confirmed the \( \alpha \) axial orientation of these protons. Similarly, the cross-peaks, observed on the NOESY spectrum, between H-29/H-19 and H-19/H-30 confirmed the \( \beta \)-axial orientation of these methyl groups (Figure S57). The cross-peaks among H-17–H-12x, H-18x, and H-21 and H-22–H-17, H-21, and H-23 in the nuclear overhauser effect spectroscopy spectrum indicated that the relative configuration at C-22 was \( \alpha \)-orientation. Hence, after extensive NMR analyses and comparison to data of gouaniaside VI (Brandao et al. 1993; Gossan et al. 2017), the aglycone of 1 was established as 16\( \beta \),22\( \beta \):16\( \alpha \),18-diepoxy-24-methylidenedammarane-3\( \beta \),20-diol.

Furthermore, the \(^1\)H NMR spectrum exhibited anomeric signals at \( \delta_H \) 4.46 (d, 7.5 Hz), 4.54 (d, 7.5 Hz), and 5.46 (d, 1.5 Hz), which indicated the presence of three sugars; the signals were correlated with carbon signals at \( \delta_C \) 105.5, 104.0, and 101.8, respectively, in the heteronuclear single quantum coherence spectrum. Each sugar proton system was characterised via \(^1\)H–\(^1\)H correlated spectroscopy, whereas glycosidic carbons were characterised via heteronuclear single quantum coherence spectroscopy. From the coupling constants and chemical shifts, two \( \beta \)-glucopyranosyl (Glc\('\) and Glc\(''\)) moieties and one \( \alpha \)-rhamnopyranosyl (Rha\(''\)) moiety were identified, respectively. The absolute configurations of the sugar residues of the acid hydrolysis of 1 were determined and compared to data in the literature (Supplementary Information).

The optical rotations of the products were compared to those of authentic \( \alpha \)-glucose \([\text{R} \, 0.30, [\chi]^D_0 = +45.8 \, (c \, 0.1, \text{H}_2\text{O})]\) and \( \alpha \)-rhamnose \([\text{R} \, 0.75, [\chi]^D_0 = -15.7 \, (c \, 0.1, \text{H}_2\text{O})]\) (Voutqueenne-Nazabadioko et al. 2013). The results indicated the presence of \( \alpha \)-glucose and \( \alpha \)-rhamnose in the acid hydrolysis products of 1, which was confirmed by the results of thin-layer chromatography. The HMBCs between \( \delta_H \) 4.46 (Glc-1\('\)) and \( \delta_H \) 89.8 (C-3), \( \delta_H \) 5.46 (Rha-1\(''\)) and \( \delta_C \) 77.9 (Glc-2\('\)), and \( \delta_H \) 4.54 (Glc-1\(''\)) and \( \delta_C \) 88.6 (Glc-3\('\)) showed that the triglycosidic chain \( \beta \)-D-glucopyranosyl-(1→3)-[\( \alpha \)-L-rhamnopyranosyl-(1→2)]-\( \beta \)-D-glucopyranosid was linked to aglycon at C-3. Based on these results, the structure of 1 was identified as 16\( \beta \),22\( \beta \):16\( \alpha \),18-diepoxy-24-methylidenedammarane-3\( \beta \),20-diol-3-O-\( \beta \)-D-glucopyranosyl-(1→3)-[\( \alpha \)-L-rhamnopyranosyl-(1→2)]-\( \beta \)-D-glucopyranoside and named gouaniaside VII.

Compound 2 was isolated as a white powder. Its molecular formula \((\text{C}_{54}\text{H}_{88}\text{O}_{22})\) was determined via HR-ESI-MS. The spectrum exhibited a molecular ion peak at \( m/z \) 1111.5663 \([\text{M} + \text{Na}]^+ \) (calculated for \( \text{C}_{54}\text{H}_{88}\text{O}_{22}\text{Na}^+ \), 1111.5659). The NMR spectra of 2 resembled those of 1, except for the presence of an additional \( \beta \)-D-xylopyranosyl moiety (Perera et al. 1993). Signals at \( \delta_H \) 4.28 (d, \( J = 7.5 \, \text{Hz} \)) and \( \delta_C \) 105.2 corresponded to anomeric proton and carbon. The deshielded signal of Glc-6\(''\) (\( \delta_C \) 70.5) indicated that the xylopyranoside moiety was attached to C-6\(''\)Glc. This was confirmed by the HMBC between H-1\(''\) \( _{\text{Xyl}} \) (\( \delta_H \) 4.28) and C-6\(''\)Glc (\( \delta_C \) 70.5) (Figures S21 and S58). Thus, the structure of 2 was determined as 16\( \beta \),22\( \beta \):16\( \alpha \),18-diepoxy-24-methylidenedammarane-3\( \beta \),20-diol-3-O-\( \beta \)-D-xylopyranosyl-(1→6)-\( \beta \)-D-glucopyranosyl-(1→3)-[\( \alpha \)-L-rhamnopyranosyl-(1→2)]-\( \beta \)-D-glucopyranoside (gouaniaside VIII).

Compound 3 was isolated as a white powder. Its molecular formula \((\text{C}_{55}\text{H}_{91}\text{O}_{23})\) was determined via HR-ESI-MS. The spectrum exhibited a quasi-molecular ion peak at \( m/z \) 1153.5464 \([\text{M} + \text{Cl}]^+ \) (calculated for \( \text{C}_{55}\text{H}_{91}\text{O}_{23}\text{Cl}^+ \), 1153.5567). The \(^1\)H- and \(^{13}\)C-NMR spectra of 3 agreed well with those of 1, except for the presence of an additional \( \beta \)-D-glucopyranosyl moiety (see Table S1) at C-6\(''\)Glc. The presence of the moiety was
indicated by the HMBC between H-1′′′ Glc (δ_H 4.34) and C-6′′′ Glc (δ_C 70.2) and the downfield-shifted signal of C-6′′′ Glc at δ_C 70.2 (Figures S35 and S59). Therefore, the structure of 3 was established as 16β,22α:16α,20-diol-3-O-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→3)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (gouaniaside IX).

Compound 4 was obtained as a white powder. Its molecular formula (C_{49}H_{82}O_{19}) was determined via HR-ESI-MS (m/z 1009.5068 [M+Cl]). The 1H and 13C NMR spectra of 4 resembled those of 1, except for the presence of D ring signals. The absence of signals at δ_C 66.8 and 38.9, corresponding to C-18 and C-15 in the structure of 1, respectively, and the presence of one methyl group (δ_H 0.99, δ_C 10.0) and one hydroxymethine group (δ_H 3.96, δ_C 77.6), suggested that the D ring was arranged differently from that in 1. The presence of upfield-shifted signals at C-14 (~3.5 ppm), C-16 (~9.2 ppm), and C-22 (~6.2 ppm) indicated that 4 had a comparable structure with the aglycone of joazeiroside B (Renault et al. 1997). The relative configuration of 4 was proposed based on the comparison of their NMR data and the agreement of the biosynthetic pathway of 1–3. Furthermore, the NMR data suggested that 4 possessed the same trisaccharide as 1 (Table S1). From these results, the structure of 4 was determined as 16,22-epoxy-24-methylidenedammarane-3β,15α,16α,20β-tetrol-3-O-β-D-glucopyranosyl-(1→3)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (Joazeiroside C).

To determine the cytotoxicity of compounds 1–3, we treated RAW 264.7 cells with serially diluted single-molecule concentrations of the compounds for 3 days and verified the number of cells in the culture via MTT analyses (Vinh et al. 2020). The results (data not shown) revealed that compounds 1–2 exhibited no cytotoxicity at a concentration of 5 μM, whereas compound 3 showed weak cytotoxicity at 25 μM.

NO is a key mediator of inflammatory response and pathogenesis (Nguyen et al. 2019). We examined the inhibitory activity of isolated compounds 1–3 against the activation of RAW 264.7 macrophages for NO production. RAW 264.7 macrophages were treated with any of compounds 1–3 for 24 h and then incubated with lipopolysaccharide. The level of NO produced by the cells decreased in a concentration-dependent manner. All tested compounds (1–3) showed strong inhibitory activity against NO production in a dose-dependent manner (Figure S61).

In conclusion, we identified four new triterpenoid glycosides from 96% EtOH extracts of *G. leptostachya*. Their structures were characterised via one-dimensional and two-dimensional NMR spectroscopy and HR-ESI-MS. *G. leptostachya* is rich in saponin. The cytotoxicity and NO inhibition result also provide a scientific rationale for further investigations of anti-inflammatory activity for this valuable medicinal plant.

### 3. Experimental

#### 3.1. Plant material

The aerial parts of *Gouania leptostachya* DC. (Rhamnaceae) were collected from Thai Nguyen province, Vietnam in November 2015. The plant was identified by Assoc. Prof. Pham Thanh Huyen (NIMM) and a voucher specimen (TB-10663C) has been deposited in the Herbarium of the Department of Medicinal Material Resources, NIMM (Hanoi, Vietnam).
3.2. Extraction and isolation

Powdered aerial parts of *G. leptostachya* (1.5 kg) were mixed with 96% EtOH (10 L × 3), and the mixture was left at room temperature for 4 days. The extract was filtered and concentrated under reduced pressure using a rotary evaporator at 40–50 °C, and a greenish-brown residue (148.0 g) was obtained. The residue was suspended in distilled water and successively fractionated with solvents of increasing polarity to obtain *n*-hexane (17.0 g), ethyl acetate (38.0 g), and *n*-butanol (21.0 g) fractions and a water layer.

The *n*-butanol fraction (20.0 g) was chromatographed on a silica gel column and eluted with a solvent system of ethyl acetate-methanol-water (6:1:0.2 → 2:1:0.2, v/v/v) to obtain five subfractions (B1–B5). Subfraction B3 (5.1 g) was separated via Diaion HP-20 column chromatography (CC) and successively eluted with water, EtOH (96%), and acetone to obtain two subfractions (B3.1 and B3.2). Subfraction B3.1 (3.5 g) was separated via reverse-phase CC with a C18 column with methanol-water (1:2 → 1:1, v/v) to obtain compounds 1 (100 mg) and 4 (12 mg) and eight subfractions (B3.1.1–B3.1.8). Subfraction B3.1.8 (0.5 g) was purified via silica gel CC with chloroform-methanol-water (3:8:1:0.1, v/v/v) as the mobile phase to obtain compounds 2 (36 mg) and 3 (21 mg).

The ethyl acetate fraction (35.0 g) was separated via silica gel CC with dichloromethane-methanol (30:1 → 5:1, v/v) as the gradient-elution solvent to obtain eight subfractions (E1–E8). Subfraction E2 (7.2 g) was separated into six subfractions (E2.1–E2.6) via silica gel CC and elution with dichloromethane-methanol (40:1, v/v). Subfraction E2.3 (0.4 g) was separated via silica gel CC with *n*-hexane-ethyl acetate-methanol (6:1:0.4, v/v/v) as the mobile phase to obtain compound 5 (10 mg).

3.2.1. Gouaniaside VII (1)
White solid; ¹H-NMR (500 MHz, CD3OD) and ¹³C-NMR (125 MHz, CD3OD): see Table S1. HR-ESI-MS m/z 957.5411 [M + H]⁺ (calcd. for C₄₉H₈₁O₁₈⁺, 957.5417).

3.2.2. Gouaniaside VIII (2)
White solid, ¹H-NMR (500 MHz, CD3OD) and ¹³C-NMR (125 MHz, CD3OD): see Table S1. HR-ESI-MS m/z 1111.5665 [M + Na]⁺ (calcd. for C₅₄H₈₈O₂₂Na⁺, 1111.5659).

3.2.3. Gouaniaside IX (3)
White solid, ¹H-NMR (500 MHz, CD3OD) and ¹³C-NMR (125 MHz, CD3OD): see Table S1. HR-ESI-MS m/z 1153.5464 [M + Cl]⁻ (calcd. for C₅₅H₉₀O₂₃Cl⁻, 1153.5567).

3.2.4. Joazeiroside C (4)
White solid, ¹H-NMR (500 MHz, CD3OD) and ¹³C-NMR (125 MHz, CD3OD): see Table S1. HR-ESI-MS m/z 1009.5068 [M + Cl]⁻ (calcd. for C₄₉H₈₂O₁₉Cl⁻, 1009.5144).

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Disclosure statement

No potential conflict of interest was reported by the authors.

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