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BENZYL AND MEGASTIGMANE GLUCOSIDES FROM THE LEAVES OF *ZANTHOXYLUM RHETSA* (ROXB.) DC. AND THEIR INHIBITORY EFFECTS ON NITRIC OXIDE PRODUCTION

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Summary

Six metabolites (**1**–**6**) were successfully isolated from the leaves of *Zanthoxylum rhetsa* using a combination of chromatographic techniques. Their structures were elucidated as gnetifolin K (**1**), 4'-*O*-*β*-D-glucopyranosyl-(*E*)-4-hydroxy-3-methylbut-2-enyl benzoate (**2**), benzyl alcohol *β*-D-glucopyranoside (**3**), byzantionoside B (**4**), icariside B_5 (**5**), and (6*S*,9*R*)-roseoside (**6**) based on spectroscopic analyses, including HRESIMS, 1D and 2D NMR, and ECD data, supported by comparison with published literature. Compounds **1** and **2** are reported from the genus *Zanthoxylum* for the first time, whereas **4**–**6** are described for the first time from *Z. rhetsa*. The isolated compounds were evaluated for their inhibitory effects on lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW264.7 macrophages. Compounds **2** and **4** exhibited inhibitory activity with IC_{50} values of 38.26 and 64.98 μM, respectively, compared with dexamethasone used as the positive control (IC_{50} = 13.12 μM).

Keywords: *Zanthoxylum rhetsa;* Rutaceae*; Benzyl; Megastigmane; NO inhibitory effect*.

1. Introduction

The genus *Zanthoxylum* L., the largest genus in the family Rutaceae, comprises approximately 300 species distributed across tropical regions of East and Southeast Asia, the Americas, and Africa [1],[2],[3]. Species of *Zanthoxylum* are economically important as both spices and medicinal plants and are widely used worldwide. Their young shoots, seeds, and fruit peels are consumed as food. The seeds are used for edible oil production, while the bark is extensively employed in traditional medicine [2],[4]. Currently, there is no accurate data regarding the exact number of *Zanthoxylum* species occurring in Vietnam. However, published studies have recorded the presence of 11–13 species in the country [5]. Traditionally, species of this genus have been used in folk medicine for promoting blood circulation, relieving blood stasis, regulating qi and alleviating pain, dispelling wind and dredging collaterals, detoxification, reducing swelling, and relieving itching. They have also been employed in the treatment of various ailments, including urinary tract infections, gynecological disorders, vomiting, diarrhea, hernia, stomachache, toothache, rheumatic arthralgia, abdominal pain, and snakebite [2],[4].

Zanthoxylum rhetsa (Roxb.) DC. is distributed across Bangladesh, China, India, Indonesia, Malaysia, Thailand, and Vietnam [6],[7]. In Vietnam, this species is native to the northwestern region, where it has long been used in traditional medicine to treat toothaches, abdominal pain, and improve digestion [8]. Previous studies have demonstrated that extracts and constituents from *Z. rhetsa* possess a wide range of biological activities, including antidiabetic, anti-inflammatory, antimicrobial, antispasmodic, antinociceptive, antidiarrheal, astringent, and diuretic effects [6],[7],[9]. Despite these reported bioactivities, the phytochemistry of *Z. rhetsa* remains relatively limited. To date, phytochemical investigations have led to the isolation of only a limited number of secondary metabolites, including alkaloids [10],[11],[12], benzyls [10],[11],[12], coumarins [13], lignans [14], and terpenoids [13]. As part of our ongoing phytochemical investigation of the genus *Zanthoxylum* [15],[16], we investigated the leaves of *Z. rhetsa* and successfully isolated six compounds (**1**–**6**, Fig. 1), whose structures were elucidated and evaluated for their inhibitory effects on NO production in LPS-induced RAW264.7 macrophages.

2. Materials and Methods

2.1. Chemicals and equipment

Optical rotations were determined on a JASCO P-2000 polarimeter (Jasco, Tokyo, Japan). The CD spectra were measured on a Chirascan spectrometer (Applied Photophysics, Surrey, UK). The NMR spectra were recorded on a Bruker AVANCE NEO 600 FT-NMR spectrometer with

TMS as an internal standard (Bruker BioSpin, Ettlingen, Germany). The HRESIMS was taken on an Agilent 6530 Accurate Mass Q-TOF LC/MS (Agilent Technologies, Santa Clara, California, US). Column chromatography (CC) was conducted on *silica gel*, Diaion HP-20, Sephadex LH-20, and RP-18. Preparative HPLC was performed using an Agilent 1100, J'sphere ODS H-80 column (4 μm, 250 × 20 mm), a flow rate of 6 mL/min, and a DAD detector at wavelengths of 205, 230, 254, and 280 nm. An isocratic mobile phase with a flow rate of 3.0 mL/min was used in pre-HPLC. Analytical thin-layer chromatography (TLC) was performed on precoated *silica gel* 60 F_{254} and RP-18 $F_{254}S$ plates (Merck, Darmstadt, Germany), and compounds were visualized by spraying with 10% H_2SO_4 in water and then heating for 1.5–2 minutes.

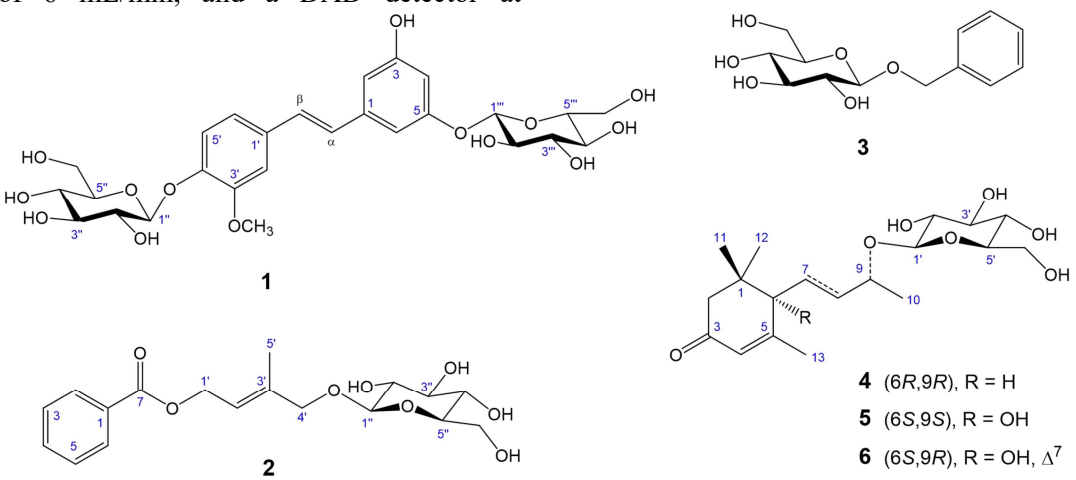


Fig. 1. Chemical structures of compounds **1**–**6**.

2.2. Plant material

The leaves of *Zanthoxylum rhetsa* were collected in Quang Tri Province, Vietnam, in December 2023 and taxonomically identified by Dr. Le Tuan Anh, Vietnam National Museum of Nature, VAST. Voucher specimens (NCCT-P173) were kept at the Institute of Chemistry, VAST.

2.3. Extraction and isolation

The dried leaves (3.2 kg) of *Z. rhetsa* were extracted with MeOH (3 × 5 L) by exhaustive maceration at room temperature for 3 days. The combined extract was concentrated under reduced pressure to afford a crude MeOH extract (250 g), which was suspended in H_2O (2.0 L) and successively partitioned with *n*-hexane (2.0 L) and EtOAc (2.0 L), to afford *n*-hexane (H, 50.1 g), EtOAc (E, 130.2 g), and aqueous (W) fractions.

The aqueous fraction was subjected to Diaion HP-20 CC, washed with H_2O (1.0 L), and eluted with MeOH (1.5 L) to yield four fractions (W1–W4). Fraction W3 (4.1 g) was further fractionated by *silica gel* CC using CH_2Cl_2-MeOH (10:1, v/v) as the eluent to afford four subfractions (W3.1–W3.4). Subfraction W3.2 (1.1 g) was chromatographed over RP-18 CC using MeOH-H_2O (1:2, v/v) to give five subfractions (W3.2.a to W3.2e). Subfraction W3.2.a (85 mg) was purified by semi-preparative RP-HPLC with 72% ACN in H_2O to afford compounds **1** (6.4 mg, t_R = 40.8 min) and **3** (5.7 mg, t_R = 54.3 min). Similarly, compounds **2** (5.1 mg, t_R = 34.6 min) and **4** (4.5 mg, t_R = 47.0 min) were purified from subfraction W3.2.b (96.2 mg) by semi-preparative RP-HPLC (ACN-H_2O containing 0.05% TFA, 70:30, v/v). Purification of subfraction W3.2.c (73.8 mg) under the same RP-HPLC conditions afforded compound **5** (6.7 mg, t_R = 33.8 min). Finally, subfraction W3.2.d (112 mg) was further purified by semi-preparative RP-HPLC using 25% ACN in H_2O to yield compound **6** (7.3 mg, t_R = 42.7 min).

Gnetifolin K (1): Cream-colored powder; $[\alpha]_D^{24}$ +92.0 (*c* 0.15, MeOH); ^{1}H (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD) spectroscopic data, see Table 1; HRESIMS *m/z* 583.2030 $[M + H]^+$ (calcd. for $[C_{27}H_{35}O_{14}]^+$, 583.2027).

4'-O-β-D-Glucopyranosyl-(E)-4-hydroxy-3-methyl but-2-enyl benzoate (2): White amorphous powder; $[\alpha]_D^{24}$ +48.6 (*c* 0.15, MeOH); ^{1}H (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD) spectroscopic data, see Table 1; HRESIMS *m/z* 391.1365 $[M + Na]^+$ (calcd. for $[C_{18}H_{24}NaO_8]^+$, 391.1368).

Benzyl alcohol β-D-glucopyranoside (3): White amorphous powder; $[\alpha]_D^{24}$ +49.7 (*c* 0.15 MeOH); ^{1}H NMR (600 MHz, CD_3OD) δ (ppm): 7.44 (2H, dd, *J* = 8.4, 1.2 Hz, H-2/H-6), 7.34 (2H, dd, *J* = 8.4, 8.4 Hz, H-3/H-5), 7.27 (1H, dd, *J* = 8.4, 1.2 Hz, H-4), 4.69 (1H, d, *J* = 12.0 Hz, H_a-7), 4.95 (1H, d, *J* = 12.0 Hz, H_b-7), 4.38 (1H, d, *J* = 7.8 Hz, H-1'), 3.36 (1H, dd, *J* = 9.0, 7.8 Hz, H-2'), 3.33 (1H, dd, *J* = 9.0, 9.0 Hz, H-3'), 3.35 (1H, t, *J* = 9.0 Hz, H-4'), 3.34 (1H, m, H-5'), 3.71 (1H, dd, *J* = 12.0, 5.4 Hz, H_a-6'), and 3.91 (1H, dd, *J* = 12.0, 2.4 Hz, H_b-6'); ^{13}C NMR (150 MHz, CD_3OD) δ (ppm): 139.1 (C-1), 129.2 (C-2/C-6), 129.3 (C-3/C-5), 128.7 (C-4), 71.7 (C-7), 103.3 (C-1'), 75.1 (C-2'), 78.1 (C-3'), 71.7 (C-4'), 78.0 (C-5'), and 62.8 (C-6').

Byzantionoside B (4): White amorphous powder; $[\alpha]_D^{24}$ +32.5 (*c* 0.15, MeOH); ECD λ_{max} ($\Delta\varepsilon$) 235 (+2.85), 332 (+1.03) nm; ^{1}H (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD) spectroscopic data, see Table 2; HRESIMS *m/z* 395.2038 $[M + Na]^+$ (calcd. for $[C_{19}H_{32}NaO_7]^+$, 395.2040).

Icariside B$_5$ (5): White amorphous powder; $[\alpha]_D^{24}$ –79.7 (*c* 0.15 MeOH); ECD λ_{max} ($\Delta\varepsilon$) 219 (+10.87), 251 (–7.15), 325 (+2.06) nm; ^{1}H (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD) spectroscopic data, see Table 2; HRESIMS *m/z* 389.2176 $[M + H]^+$ (calcd. for $[C_{19}H_{33}O_8]^+$, 389.2170), 411.2001 $[M + Na]^+$ (calcd. for $[C_{19}H_{32}NaO_8]^+$, 411.1989), and 406.2441 $[M + NH_4]^+$ (calcd. for $[C_{19}H_{32}O_8NH_4]^+$, 406.2435).

(6S,9R)-Roseoside (6): White amorphous powder; $[\alpha]_D^{24}$ +81.3 (*c* 0.15, MeOH); ECD λ_{max} ($\Delta\varepsilon$) 205 (–4.75), 241 (+8.05), 323 (–1.01) nm; ^{1}H NMR (600 MHz, CD_3OD) δ (ppm): 2.15 (1H, d, *J* = 16.8 Hz, H_a-2), 2.51 (1H, d, *J* = 16.8 Hz, H_b-2), 5.87 (1H, br s, H-4), 5.85 (1H, m, H-7), 5.87 (1H, m, H-8), 4.42 (1H, m, H-9), 1.29 (3H, s, H-10), 1.03 (3H, s, H-11), 1.02 (3H, s, H-12), 1.92 (3H, s, H-13), 4.34 (1H, d, *J* = 7.8 Hz, H-1'), 3.17 (1H, dd, *J* = 9.0, 7.8 Hz, H-2'), 3.26 (1H, dd, *J* = 9.0, 9.0 Hz, H-3'), 3.33 (1H, t, *J* = 9.0 Hz, H-4'), 3.22 (1H, m, H-5'), 3.62 (1H, dd, *J* = 12.0, 5.4 Hz, H_a-6'), and 3.85 (1H, dd, *J* = 12.0, 1.8 Hz, H_b-6'); ^{13}C NMR (150 MHz, CD_3OD) δ (ppm): 42.2 (C-1), 50.7 (C-2), 201.2 (C-3), 127.2 (C-4), 167.2 (C-5), 80.8 (C-6), 131.6 (C-7), 135.3 (C-8), 77.3 (C-9), 21.2 (C-10), 23.4 (C-11), 23.7 (C-12), 19.6 (C-13), 102.8 (C-1'), 75.3 (C-2'), 78.2 (C-3'), 71.7 (C-4'), 78.0 (C-5'), and 62.8 (C-6'); HRESIMS *m/z* 409.1850 $[M + Na]^+$ (calcd. for $[C_{19}H_{30}NaO_8]^+$, 409.1836).

2.4. NO inhibitory assay

Nitric oxide assay was performed as previously described [17],[18]. In brief, RAW264.7 cells, originally sourced from the American Type Culture Collection (ATCC, Manassas, VA, USA), were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco-BRL, St. Louis, MO, USA) supplemented with 5% heat-inactivated fetal bovine serum, along with 100 U/mL penicillin and 100 μg/mL streptomycin. The cultures were incubated under controlled conditions at 37°C in a humidified atmosphere containing 5% CO_2. Cells were seeded in a 96-well plate at a density of 2×10^5 cells/well and allowed to adhere for 6 h. The cells were then treated with the test compounds (10–100 μM) or vehicle control, followed 2 h later by LPS (1 μg/mL). After incubation under standard culture conditions (37°C, 5% CO_2), nitrite accumulation in the culture medium was quantified using the Griess reaction. To assess cell viability, the remaining adherent cells were subjected to the MTT assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma Chemical Co., St. Louis, MO, USA), and absorbance was measured at 570 nm. Culture medium (100 μL) was mixed with an equal volume of Griess reagent and incubated at room temperature for 10 minutes. Absorbance was measured at 540 nm on a microplate reader. Nitrite concentration, as an indicator of NO production, was determined using a standard curve that was built from $NaNO_2$ serial diluted solutions. Experiments were performed in triplicate. The IC_{50} values were generated by TableCurve 2Dv4 software.

3. Results and Discussion

Compound **1** was purified as a cream-colored powder. Its molecular formula was determined to be $C_{27}H_{34}O_{14}$, corresponding to 11 degrees of unsaturation, based on its protonated molecular ion at *m/z* 583.2030 $[M + H]^+$ (calcd. for $[C_{27}H_{35}O_{14}]^+$, 583.2027) observed in the HRESIMS together with the ^{13}C NMR data. The ^{1}H NMR data of **1** displayed six aromatic proton signals, comprising an A_2B-spin system attributable to a 1,3,5-trisubstituted benzene ring [δ_H 6.66 (H-2), 6.50 (H-4), and 6.85 (H-6), each 1H, t, *J* = 1.2 Hz] and an ABX-spin system characteristic of a 1,3,4-trisubstituted benzene ring [δ_H 7.31 (1H, d, *J* = 1.8 Hz, H-2'), 7.16 (1H, d, *J* = 8.4 Hz, H-5'), and 7.10 (1H, dd, *J* = 8.4, 1.8 Hz, H-6')]. The spectrum also showed signals

attributable to two *trans*-olefinic protons [δ_H 6.98 (H-α) and 7.07 (H-β), (1H, d, J = 16.2 Hz) each], one methoxy group [δ_H 3.94 (3H, s, 3'-OCH_3)], and two anomeric protons [δ_H 4.94 (H-1") and 4.92 (H-1'"), each 1H, d, J = 7.8 Hz]. The β-configurations of sugar moieties were deduced from the ^3J value of the anomeric protons. The ^{13}C NMR spectrum of **1** in conjunction with the HSQC spectrum revealed 27 carbon resonance, which could be classified into 12 aromatic carbons (δ_C 104.5-160.5), two olefinic carbons [δ_C 128.6 and 129.7], and one methoxy group (δ_C 56.8), and 12 signals attributable to two β-D-glucopyranosyl units (δ_C 102.7, 74.9, 78.1, 71.3, 78.3, 62.5, 102.4, 75.0, 77.8, 71.5, 78.2, and 62.6) (Table 1). These spectroscopic features, combined with the molecular formula, indicated that compound **1** was a stilbene diglucoside [19]. Detailed analysis of the 2D NMR spectra enabled complete assignment of all proton and carbon resonances. The ^1H–^1H COSY spectrum established two aromatic spin systems corresponding to 1,3,5-trisubstituted and 1,3,4-trisubstituted benzene rings, while the HMBC spectrum confirmed a *trans*-stilbene framework (Fig. 2). The methoxy group was assigned to C-3' based on the HMBC correlation between the methoxy proton signal (δ_H 3.94) and C-3' (δ_C 150.9). The two β-D-glucopyranosyl units were located at C-5 and C-4' through the HMBC correlations of H-1'" (δ_H 4.92) with C-5 (δ_C 160.5) and H-1" (δ_H 4.94) with C-4' (δ_C 147.8), respectively (Fig. 2). Therefore, the 2D structure of **1** was established as shown in Fig. 2. Compound **1** was finally identified as gnetifolin K (Fig. 1 and Table 1). This compound was previously obtained from several species of the family Gnetaceae, including *Gnetum africanum* [20], *G. gnemon* [21], *G. montanum* [19],[22], and *G. latifolium* [23]. To the best of our knowledge, this is the first report of compound **1** from *Z. rhetsa* [2],[4].

Table 1. NMR spectroscopic data of compounds **1** and **2** and the reference compounds.

No.	$^{\#}\delta_C$[a]	**1**		$^{\$}\delta_C$[a]	**2**	
		δ_C[a,b]	δ_H[a,c] mult. (J in Hz)		δ_C[a,b]	δ_H[a,c] mult. (J in Hz)
1	147.7	147.8	-	131.7	131.6	-
2	108.7	108.6	6.66 t (1.2)	130.6	130.5	8.03 dd (8.4, 1.2)
3	159.9	159.6	-	129.7	129.6	7.49 dd (8.4, 8.4)
4	104.6	104.5	6.50 t (1.2)	134.4	134.2	7.61 dd (8.4, 1.2)
5	160.4	160.5	-	129.7	129.6	7.49 dd (8.4, 8.4)
6	107.2	107.2	6.85 t (1.2)	130.6	130.5	8.03 dd (8.4, 1.2)
7				168.1	168.0	-
α	128.6	128.6	6.98 d (16.2)			
β	129.6	129.7	7.07 d (16.2)			
1'	133.9	133.9	-	62.5	62.4	4.93 br d (6.6)
2'	121.1	121.1	7.31 d (1.8)	122.3	122.1	5.85 m
3'	150.9	150.9	-	139.6	139.5	-
4'	147.7	147.8	-	74.6	74.5	4.33 d (12.6) 4.13 d (12.6)
5'	117.9	117.9	7.16 d (8.4)	14.5	14.3	1.86 s
6'	111.4	111.4	7.10 dd (8.4, 1.8)			
3'-OCH_3	56.8	56.8	3.94 s			
1"	102.3	102.7	4.94 d (7.8)	103.3	103.2	4.30 d (7.8)
2"	74.8	74.9	3.53 dd (9.0, 7.8)	75.2	75.1	3.23 dd (9.0, 7.8)
3"	77.9	78.1	3.47*	78.2	78.2	3.30 dd (9.0, 9.0)
4"	71.3	71.3	3.42*	71.8	71.7	3.36 t (9.0)
5"	78.1	78.3	3.40 m	78.2	78.0	3.25 m
6"	62.5	62.5	3.72 dd (12.0, 5.4) 3.91 dd (12.0, 2.4)	62.9	62.8	3.68 dd (12.0, 4.8) 3.87 dd (12.0, 2.4)
1'"	102.4	102.4	4.92 d (7.8)			
2'"	74.9	75.0	3.47 dd (9.0, 7.8)			
3'"	77.9	77.8	3.46*			
4'"	71.4	71.5	3.41*			
5'"	78.0	78.2	3.43 m			
6'"	62.6	62.6	3.74 dd (12.0, 5.4) 3.96 dd (12.0, 2.4)			

Measured in [a]CD_3OD, [b]150 MHz, and [c]600 MHz. *Overlapped signals, $^{\#}\delta_C$ of gnetifolin K [19], and $^{\$}\delta_C$ of (*E*)-4-hydroxy-3-methylbut-2-enyl benzoate 4-*O*-β-D-glucoside [24].

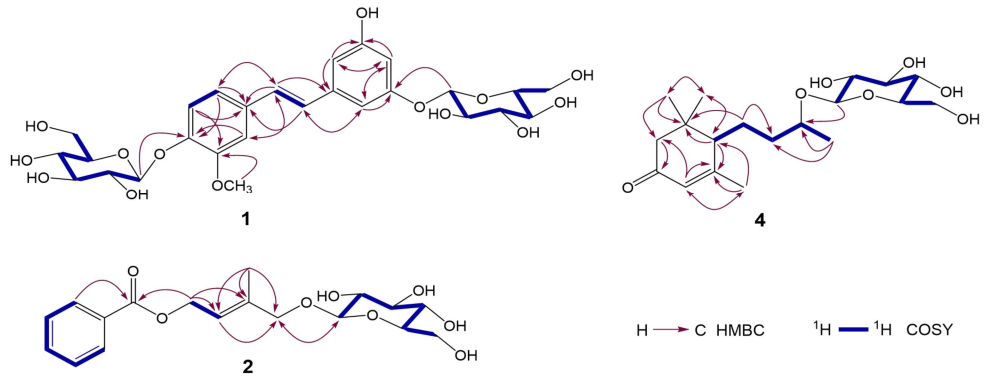


Fig. 2. Key HMBC and ^{1}H–^{1}H COSY correlations of compounds **1**, **2**, and **4**

Compound **2** was isolated as a white amorphous powder. The HRESIMS data of **2** showed a sodium adduct ion at *m/z* 391.1365 $[M + Na]^+$ (calcd. for $[C_{18}H_{24}NaO_8]^+$, 391.1368), consistent with the molecular formula $C_{18}H_{24}O_8$ (seven degrees of unsaturation). Analysis of the $^{1}H/^{13}C$ NMR spectroscopic data (Table 1) revealed the presence of a benzoic acid moiety [δ_C 131.6 (C-1), 130.5 (C-2/C-6)/δ_H 8.03 (2H, dd, J = 8.4, 1.2 Hz, H-2/H-6), 129.6 (C-3/C-5)/δ_H 7.49 (2H, dd, J = 8.4, 8.4 Hz, H-3/H-5), 134.2 (C-4)/δ_H 7.61(1H, dd, J = 8.4, 1.2 Hz, H-4), and 168.0 (C-7)] and a sugar unit [δ_C 103.2 (C-1"), 75.1 (C-2"), 78.2 (C-3"), 71.7 (C-4"), 78.0 (C-5"), and 62.8 (C-6")]. The glucose moiety was further supported by the presence of an anomeric proton [δ_H 4.30 (1H, d, J = 7.8 Hz, H-1")] and an oxygenated methylene group [δ_H 3.68 (1H, dd, J = 12.0, 4.8 Hz, H_a-6")/3.87 (1H, dd, J = 12.0, 2.4 Hz, H_b-6"). The large coupling constant of the anomeric proton indicated a β-glucosidic linkage. Besides, the NMR data displayed signals attributable to two oxygenated sp^3 methylene carbons [δ_C 62.4 (C-1')/δ_H 4.93 (2H, br d, J = 6.6 Hz, H-1') and 74.5 (C-4')/δ_H 4.33 (1H, d, J = 12.6 Hz, H_a-4'), 4.13 (1H, d, J = 12.6 Hz, H_b-4')], one olefinic methine carbon [δ_C 122.1 (C-2')/δ_H 5.85 (1H, m, H-2')], one non-protonated carbon [δ_C 139.5 (C-3')], and one methyl group [δ_C 14.3 (C-5')/δ_H 1.86 (3H, s, H-5')], suggesting the presence of a 2-methylbut-2-en-1,4-diol unit [24]. Three spin systems, C-2–C-6, C-1'–C-2', and C-1"–C-6" were established from the ^{1}H–^{1}H COSY spectrum and while the remaining connectivities were assigned from the HMBC spectrum (Fig. 2). Key HMBC correlations from H-1' to C-2', and C-3', from H-2' to C-1', C-3', and C-4', and from H-5' to C-2', C-3', and C-4' confirmed the 2'-methylbut-2'-en-1',4'-diol fragment. Additional HMBC correlations from H-2/H-6 to C-4 and C-7, and from H-3/H-5 to C-1 supported assignment of the benzoate moiety. The ester linkage between the benzoate and 2'-methylbut-2'-en-1',4'-diol fragment was established by the HMBC correlation from H-1' to C-7, establishing esterification between the 2'-methylbut-2'-en-1',4'-diol unit and benzoic acid moiety, whereas the correlation from H-1" to C-4' located the β-D-glucopyranosyl unit at C-4'. Accordingly, compound **2** was identified as 4'-*O*-β-D-glucopyranosyl-(*E*)-4-hydroxy-3-methylbut-2-enyl benzoate by comparison of its spectroscopic data with those reported in the literature [24]. This compound was previously reported from *Artemisia scoparia* [24] but is reported here for the first time from *Zanthoxylum rhetsa* [2],[4].

Compound **4** was obtained as a white amorphous powder. Its molecular formula was established as $C_{19}H_{32}O_7$ based on the HRESIMS sodium adduct ion at *m/z* 395.2038 $[M + Na]^+$ (calcd. for $[C_{19}H_{32}NaO_7]^+$, 395.2040). The ^{1}H NMR spectrum of **4** exhibited four methyl groups [δ_H 1.20 (d, J = 6.0 Hz, H-10), 1.03 (s, H-11), 1.11 (s, H-12), and 2.07 (s, H-13), each 3H], one methine [δ_H 1.97 (1H, m, H-6)], one oxymethine [δ_H 3.90 (1H, m, H-9)], one methylene [δ_H 1.98 (1H, d, J = 16.8 Hz, H_a-2) and 2.48 (1H, d, J = 16.8 Hz, H_b-2)], one trisubstituted double bond [δ_H 5.82 (1H, s, H-4)], and an anomeric proton [δ_H 4.35 (1H, d, J = 8.4 Hz, H-1')]. A series of partially overlapping multiplet signals of aliphatic methylene protons (δ_H 1.52−1.99) were also indicated in the ^{1}H NMR spectrum. The ^{13}C NMR data and HSQC spectra of **4** confirmed 19 carbon signals, comprising 13 carbons attributable to a megastigmane aglycone and six carbons belonging to a hexose unit. The aglycone carbon signals were consistent with α,β-unsaturated carbonyl functionalities [δ_C 202.4 (C-3), 125.4 (C-4), and 170.1 (C-5)], two methines [one oxygenated at δ_C 75.5 (C-9)], three methylenes [δ_C 48.1 (C-2), 26.8 (C-7), and 37.8 (C-8)], four

methyl groups [δ_C 19.9 (C-10), 29.1 (C-11), 27.5 (C-12), and 25.0 (C-13)], and one non-protonated carbon [δ_C 37.3 (C-1)]. The sugar moiety was characterized by an anomeric carbon (δ_C 102.1), and five oxygenated carbons (δ_C 75.2, 78.3, 71.9, 77.9, and 62.9), as well as an anomeric proton [δ_H 4.35 (1H, d, J = 8.4 Hz, H-1')], oxymethine protons (δ_H 3.16−3.57), a pair of oxymethylene protons [δ_H 3.67 (1H, dd, J = 11.4, 5.4 Hz) and 3.87 (1H, dd, J = 11.4, 2.4 Hz)], indicated the presence of a glucopyranosyl moiety in **4** [25]. Furthermore, the coupling constant (J = 8.4 Hz) of the anomeric proton indicated a β-form for the glucopyranose moiety, which allowed the monosaccharide unit to be confirmed as β-D-glucopyranose [25]. The position of the monosaccharide unit was confirmed as being linked at C-9 by the HMBC correlation of H-1' (δ_H 4.35) with C-9 (δ_C 75.5). Analysis of the ^{1}H–^{1}H COSY spectrum of **4** confirmed two proton spin-systems corresponding to the C-6–C-10 and C-1'–C-6' fragments, while the remaining connectivities were established by the HMBC spectrum. Key HMBC correlations from H_3-11, H_3-12 to C-1, C-2, C-6; from H-6 to C-1, C-5, C-7; from H-2 to C-1, C-3, C-6 confirmed that C-2, C-6, C-11, and C-12 were all linked with C-1. Additional HMBC correlations from H-4 to C-2, C-3, from H-2 to C-3 confirmed the connection of C-3 with C-2, C-4, H_3-13 with C-4, C-5, C-6 confirmed a cyclohex-4-en-3-one ring. Combined analysis of the ^{1}H–^{1}H COSY and HMBC spectra (Fig. 2) established a megastigman-4-en-3-one-9-ol skeleton with a β-D-glucopyranosyl unit attached at C-9. Comparison of its NMR spectroscopic data (Table 2) with those reported in the literature identified **4** as byzantionoside B [25].

The relative configuration was supported by the NOESY correlation between H-6 and H_3-12, indicating that these protons were cofacial. In addition, the ^{13}C chemical shift at C-9 (δ_C 75.5) of **4** closely matched that of the 9*R*-isomer (δ_C 75.5) of byzantionoside B [25], and differed significantly from that of the 9*S*-isomer (δ_C 77.7) of blumenol C glucoside [25], supporting the assignment of the 9*R* configuration. The absolute configuration of compound **4** was further established as 6*R*,9*R* based on the positive Cotton effects (CEs) at 235 and 332 nm in the ECD spectrum, which closely matched those reported for byzantionoside B [(6*R*,9*R*): positive CEs at 235 and 332 nm] [25]. Based on the results obtained, compound **4** was elucidated as (6*R*,9*R*)-megastigman-4-en-3-one 9-*O*-β-D-glucopyranoside (byzantionoside B).

Three remaining compounds were identified as benzyl alcohol β-D-glucopyranoside (**3**) [27], icariside B_5 (**5**) [26], and (6*S*,9*R*)-roseoside (**6**) [28] by comparison of their physical and spectroscopic data to those reported in the references.

Table 2. NMR spectroscopic data of compounds **4** and **5** and the reference compounds

No.	$^@\delta_C$[a]	**4**		$^\&\delta_C$[a]	**5**	
		δ_C[a,b]	δ_H[a,c] mult. (J in Hz)		δ_C[a,b]	δ_H[a,c] mult. (J in Hz)
1	37.4	37.3	-	43.2	43.1	-
2	48.2	48.1	1.98 d (16.8) 2.48 d (16.8)	51.2	51.1	2.16 d (18.0) 2.68 d (18.0)
3	202.5	202.4	-	201.1	201.0	-
4	125.5	125.4	5.82 br s	126.8	126.7	5.85 br s
5	170.1	170.1	-	171.8	171.7	-
6	52.5	52.4	1.97 m	79.5	79.4	-
7	26.9	26.8	1.52 m 1.99 m	34.8	34.7	1.87 dd (13.2, 3.6) 2.15 dd (13.2, 4.8)
8	37.9	37.8	1.62 m/1.68 m	33.1	33.0	1.52 m/1.80 m
9	75.7	75.5	3.90 m	77.9	78.3	3.80 m
10	19.9	19.9	1.20 d (6.0)	22.2	22.1	1.27 d (6.0)
11	29.1	29.1	1.03 s	24.5	24.4	1.04 s
12	27.6	27.5	1.11 s	24.1	24.0	1.12 s
13	25.0	25.0	2.07 s	22.0	22.0	2.06 s
1'	102.3	102.1	4.35 d (8.4)	104.4	104.3	4.34 d (7.8)
2'	75.3	75.2	3.16 dd (9.0, 8.4)	75.4	75.3	3.16 dd (9.0, 7.8)
3'	78.3	78.3	3.37 dd (9.0, 9.0)	78.4	77.8	3.27 dd (9.0, 9.0)
4'	72.0	71.9	3.29 t (9.0)	71.8	71.6	3.28 t (9.0)
5'	78.0	77.9	3.26 m	78.4	78.2	3.35 m
6'	63.1	62.9	3.67 dd (11.4, 5.4) 3.87 dd (11.4, 2.4)	62.9	62.8	3.68 dd (12.0, 5.4) 3.87 dd (12.0, 2.4)

Measured in [a]CD_3OD, [b]150 MHz, and [c]600 MHz. $^@\delta_C$ of byzantionoside B [25] and $^\&\delta_C$ of icariside B_5 [26].

Compounds **1** and **2** are reported from the genus *Zanthoxylum* for the first time [2],[4], thereby expanding the known chemical diversity of the genus. Their occurrence in *Z. rhetsa* also broadens the phytochemical profile of this species. However, additional phytochemical investigations of *Zanthoxylum* species are required to assess their potential chemotaxonomic significance. In addition, compounds **4**–**6** are described from *Z. rhetsa* for the first time [2],[4]. These findings extend the known distribution of compounds **1** and **2** within the genus *Zanthoxylum* and document compounds **4**–**6** in *Z. rhetsa* for the first time. This unexpected distribution may indicate similarities in secondary metabolite biosynthetic pathways among phylogenetically distant taxa.

Nitric oxide (NO) production and release are closely associated with various inflammatory pathophysiological processes. As a key signaling molecule in living systems, NO plays essential biological roles; however, excessive NO production has been linked to the development of inflammatory diseases [29]. Therefore, suppressing excessive NO production is considered a valuable strategy for controlling inflammatory responses. The isolated compounds were evaluated for their inhibitory effects on NO production in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages, with dexamethasone used as the positive control. None of the test compounds showed any obvious cytotoxicity at concentrations effective for NO inhibition, with cell viability over 90%. Among the isolates, compounds **2** and **4** exhibited moderate inhibitory activity against LPS-induced NO production in RAW264.7 cells, with IC_{50} values of 38.26 and 64.98 μM, respectively, compared with dexamethasone (IC_{50} = 13.12 μM). The remaining compounds did not achieve 50% inhibition at the highest concentration tested (80 μM) and were therefore recorded as IC_{50} > 80 μM (Table 3).

Table 3. Inhibitory effects of the isolated compounds on NO production induced by LPS in RAW264.7 cells.

Compound	IC_{50} (μM)[a]	Compound	IC_{50} (μM)
1	>80	**5**	>80
2	38.26 ± 1.29	**6**	>80
3	>80	Dexamethasone[b]	13.12 ± 1.12
4	64.98 ± 2.16		

[a]Values were presented as means ± SD (n=3). [b]Dexamethasone was used as a positive control. The IC_{50} values >80 μM indicate that the compounds did not achieve 50% inhibition of NO production at the highest non-cytotoxic concentration tested (100 μM).

4. Conclusion

The phytochemical investigation of the leaves of *Zanthoxylum rhetsa* resulted in the isolation of six compounds, including gnetifolin K (**1**), 4'-*O*-β-D-glucopyranosyl-(*E*)-4-hydroxy-3-methylbut-2-enyl benzoate (**2**), benzyl alcohol β-D-glucopyranoside (**3**), byzantionoside B (**4**), icariside B_5 (**5**), and (6*S*,9*R*)-roseoside (**6**). Their structures were elucidated by comprehensive spectroscopic analyses (HRESIMS, 1D and 2D NMR, and ECD data) and comparison with literature data. Among the isolates, compounds **2** and **4** exhibited moderate inhibitory activity against LPS-induced NO production in RAW264.7 macrophages, with IC_{50} values of 38.26 and 64.98 μM, respectively. To the best of our knowledge, compounds **1** and **2** are reported from the genus *Zanthoxylum* for the first time, whereas **4**–**6** are identified for the first time from *Z. rhetsa*. These findings expand the known chemical diversity of the genus and provide additional phytochemical information on *Z. rhetsa*.

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