SHORT REPORT

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A New Megastigmane Glycoside and Other Constituents from *Amomum muricarpum* Elmer

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Abstract: An updated phytochemical investigation of the aerial parts of *Amomum muricarpum* Elmer led to the isolation of a new megastigmane glycoside, *(*3*S**,5*R**,6*R**,9*R**)*-6,9-epoxy-3,5-megastigmanediol 3-*O*-rutinoside (**1**) and five known phenolic compounds. Their structures were elucidated by spectroscopic evidence including HRESIMS and NMR data. All the isolates were reported for the first time from this plant. The anti-inflammatory effect *via* the inhibition of NO production of isolated compounds was evaluated in LPS-stimulated RAW 264.7 cells. Our study found that 5,7-dimethoxyflavone **(4)** was the most active compound with the IC_{50} = 29.5 µM.

Keywords: *Amomum muricarpum;* megastigmane; 5,7-dimethoxyflavone; anti-inflammation.

1. Plant Source

The *Amomum* genus (Zingiberaceae) includes about 170 species, the second-largest one of the ginger family [1]. The plants of the genus *Amomum* distributes widely in Vietnam, China, Laos and other countries in South Asia [1, 2]. In the current study, aerial parts of *A. muricarpum* were collected in Me Linh biodiversity station, Vinh Phuc, Vietnam in May 2019 and were identified by Dr. Nguyen The Cuong, Institute of Ecology and Biological Resources, VAST. A voucher specimen (AM-01) was deposited at the University of Science and Technology of Hanoi.

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2. Previous study

While recent studies have investigated flavonoids, terpenoids, steroids and diarylheptanoids with anti-inflammatory, antibacterial, antioxidant, and anti-tumour activities from some *Amomum* species, such as *A. tsaoko, A. subulatum, A. aculeatum,* or *A. aromaticum* [2-8], just a few phytochemical papers on *A. muricarpum* Elmer have been reported [9-12]. Previously, the methanolic extract of *A. muricarpum* exhibited potent anti-inflammatory activities by suppressing excessive nitric oxide (NO) [9].

3. Present study

In the present study, a new megastigmane glycoside **(1)** and five phenolic compounds (**2**-**6**) were isolated from *A. muricarpum* (Figure 1). The the arial parts of *A. muricarpum* (4.6 kg) were extracted in methanol (16 L × 3 times) in an ultrasonic bath at 40 °C for 1 hour. The combined solution was concentrated to obtain the methanol (MeOH) extract (300 g), which was suspended in water and successively partitioned with n-hexane and ethyl acetate (EA), respectively. The organic layers were separated and evaporated to obtain hexane (75 g) and EA (42 g) residues, whereas the water layer was partly concentrated and adsorbed on a Diaion HP20 column. The column was washed with water, then eluted with 25% methanol in water and then 100% methanol to yield M50 (8.0 g) and M100 (6.1 g) fractions, respectively. Fraction M100 was separated on a silica gel CC with gradient mixtures of EA–MeOH (50/1-0/1, v/v) to yield four subfractions (Ms1-Ms4). The fraction Ms2 was purified on an RP-C18 CC eluted with MeOH 50% in water to obtain compound **1** (2.8 mg). The EA residue was subjected to a silica gel CC with gradient mixtures of CH_2Cl_2–MeOH (1/0-0/1, v/v) to yield ten fractions: E1-E10. Fraction E2 was separated on a silica gel CC eluted with n-hexane–EA (1/1, v/v) to obtain compound **2** (6.9 mg). Fraction E6 was loaded on a silica gel CC with CH_2Cl_2–MeOH (30/1, v/v) elution, followed by an RP C18 CC eluted with MeOH 75% in water to yield compound **3** (7.1 mg). Compound **4** (18.8 mg) was obtained by separating fraction E7 on a silica gel CC with n-hexane–acetone (1/1, v/v) elution. The fraction E8 was loaded on a silica gel CC with CH_2Cl_2–MeOH (10/1, v/v) elution to obtain compounds **5** (8.1 mg) and **6** (5.0 mg).

(3S,5R*,6R*,9R*)-6,9-epoxy-3,5-megastigmanediol 3-O-rutinoside **(1)***: white amorphous powder; $[\alpha]_D^{25}$ -36.8 (MeOH, *c* 0.4). HR-ESI-MS: $[M + Cl]^-$ *m/z* 571.2521 (calcd. 571.2527 for $C_{25}H_{44}O_{12}Cl$). ^{1}H NMR (500 MHz, CD_3OD) δ_H (ppm): 1.67 (1H, t, *J* = 12.0 Hz, H-2a), 1.58 (1H, m, H-2b), 4.14 (1H, m, H-3), 1.95 (1H, m, H-4a), 1.78 (1H, dd, *J* = 13.0, 11.5 Hz, H-4b), 2.11 (1H, m, H-7a), 1.92 (1H, m, H-7b), 2.05 (1H, m, H-8a), 1.48 (1H, m, H-8b), 4.13 (1H, m, H-9), 1.21 (3H, d, *J* = 6.5 Hz, H-10), 0.95 (3H, s, H-11), 1.22 (3H, s, H-12), 1.20 (3H, s, H-13), 4.39 (1H, d, *J* = 7.5 Hz, H-1′), 4.75(1H, d, *J* = 1.5 Hz, H-1"). ^{13}C NMR (125 MHz, CD_3OD) δ_C (ppm): 40.2 (C-1), 44.9 (C-2), 73.7 (C-3), 43.7 (C-4), 78 (C-5), 91.0 (C-6), 28.0 (C-7), 36.3 (C-8), 77.9 (C-9), 21.1 (C-10), 29.0 (C-11), 26.3 (C-12), 27.8 (C-13), 102.7 (C-1′), 75.0 (C-2′), 78.7 (C-3′), 71.6 (C-4′), 76.6 (C-5′), 67.8 (C-6′), 102 (C-1″), 72.1 (C-2″), 72.3 (C-3″), 73.7 (C-4″), 69.7 (C-5″), 18.1 (C-6″). ^{1}H NMR (500 MHz, Pyridine-d_5) δ_H (ppm): 2.09 (1H, t, *J* = 12.0 Hz, H-2a), 1.97 (1H, dd, *J* = 12.0, 4.0 Hz, H-2b), 4.80 (1H, m, H-3), 2.43 (1H, brd, *J* = 11.0 Hz, H-4a), 2.17 (1H, dd, *J* = 13.0, 12.0 Hz, H-4b), 2.30 (1H, m, H-7a), 1.93 (1H, m, H-7b), 1.87 (1H, m, H-8a), 1.34 (1H, m, H-8b), 4.04 (1H, m, H-9), 1.16 (3H, d, *J* = 6.5 Hz, H-10), 0.95 (3H, s, H-11), 1.47 (3H, s, H-12), 1.35 (3H, s, H-13), 4.96 (1H, d, *J* = 7.5 Hz, H-1′), 5.46 (1H, brs, H-1"). ^{13}C NMR (125 MHz, Pyridine-d_5) δ_C (ppm): 39.5 (C-1), 44.8 (C-2), 72.1 (C-3), 43.8 (C-4), 77.4 (C-5), 90.2 (C-6), 27.5 (C-7), 35.5 (C-8), 76.6 (C-9), 21.1 (C-10), 28.7 (C-11), 26.2 (C-12), 28.0 (C-13), 102.6 (C-1′), 75.2 (C-2′), 76.6 (C-3′), 71.8 (C-4′), 73.9 (C-5′), 68.5 (C-6′), 102.3 (C-1″), 72.6 (C-2″), 72.2 (C-3″), 69.5 (C-4″), 69.5 (C-5″), 18.6 (C-6″).

Sugar Identification: Compound **1** (1.5 mg) was hydrolysed in HCl 1N (1 mL) at 80 °C for 2 h. Next, the solution was partitioned with EA (1 mL × 3 times). Then, the aqueous layer was neutralised with

ammonia solution and dried under vacuum conditions. Next, the obtained residue was mixed with 200 µL of pyridine, which contained 10 µmol of L-cysteine methyl ester, and heated at 80 °C. After 1 hour, 10 µL *o*-tolyl isothiocyanate was added, and the mixture was heated for another hour. The product was injected directly into the HPLC system using a Hypersil Gold column (4.6 × 150 mm, D × L) with a mobile phase of 20% acetonitrile in 0.1% formic acid (in water, v/v), with UV detection at 254 nm. The sugars were identified as D-glucose and L-rhamnose by comparing the analyte's retention times and UV spectra to those of standard sugars under the same reacting and analysing conditions.

NO production Inhibition Assay: The effects of samples on the NO production in LPS-stimulated RAW 264.7 macrophage cells, based on the Griess reaction, were examined. The cells were seeded in a 96-well plate at the concentration of 0.5×10^5 cells per well and incubated in the humidified incubator at 37°C and 5% CO_2 for 22 hours. After incubation, cells were treated with the sample with concentrations from 3 to 25 µg/mL then 0.1 mg/mL LPS (Sigma Aldrich, USA) was added after 30 minutes. The cells were incubated for the next 24 hours. Then, 100 µL of the culture supernatant was transferred to another 96-well plate and mixed with 100 µL of Griess reagent. The absorbance of the reaction solution was read at 570 nm with an iMark microplate reader (BioRad, USA). The remaining cells from the original 96-well plate were further used for the cell viability assay (MTT assay). The assay is based on the cleaving action of dehydrogenases in functioning mitochondria of living cells on the tetrazolium ring of MTT (3-(4,5-dimethylthazol 2-yl)-2,5-diphenyl tetrazolium bromide), thus estimating the +viable cell number. Cardamonin, which is a well-known NO production inhibitor, was used as a positive control.

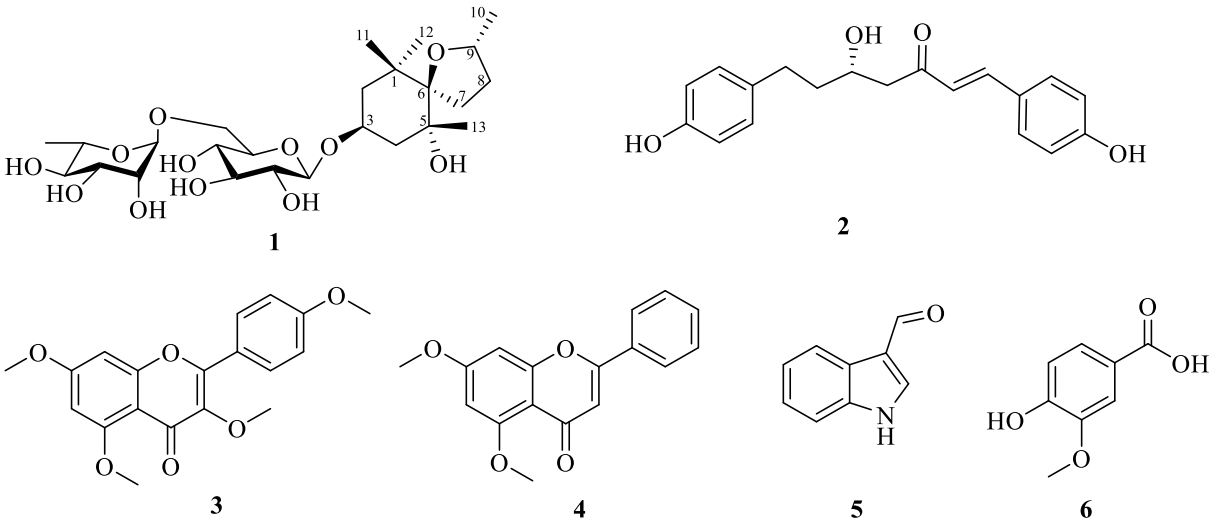

Figure 1. Structures of the isolated compounds from *A. muricarpum*

Compound **1** was isolated as a white amorphous powder from the aerial parts of *A. muricarpum.* The HR-ESI-MS spectrum (negative mode) of **1** showed the ion at *m/z* 571.2526 $[M + Cl]^-$ (calcd 571.2521 for $C_{25}H_{44}O_{12}Cl$, mass tolerance at 0.82 ppm), which suggested the molecular formula $C_{25}H_{44}O_{12}$ for **1**. The signals of two anomeric protons at δ_H 4.75 (1H, d, *J* = 1.5 Hz) and 4.39 (1H, d, *J* = 7.5 Hz) in the ^{1}H NMR spectrum indicated the presence of two sugar units. The sugar moieties were determined as D-glucose and L-rhamnose by acid hydrolysis and analysis of the derivatives of these sugars [13]. The coupling constants of H-1′ (*J* = 7.5 Hz) and H-1″ (*J* = 1.5 Hz) together with the HMBC correlation between H-1″ (δ_H 4.75) and C-6′ (δ_C 67.8) indicated the sugar linkage as *α*-L-rhamnopyranosyl-(1→6)-*β*-D-glucopyranosyl or rutinoside. Except for the signals of the sugar units, the ^{13}C NMR spectrum of compound **1** exhibited 13 signals of the aglycone, including four methyl groups, four methylene groups, two methine groups and three quaternary carbons. The chains from C-2 to C-4 and from C-7 to C-10 could be determined by the COSY correlations of the representative

protons. Two of the four methyl signals (δ_C 29.0 and 26.3) were assignable to germinal methyls (C-11 and C-12) on the quaternary carbon (C-1) at δ_C 40.2. These were confirmed by HMBC correlations from H-11 (δ_H 0.95) and H-12 (δ_H 1.22) to C-1 (δ_C 40.2). Similarly, the positions of methyl groups at C-13 and C-10 could be identified by HMBC correlations from H-13 (δ_H 1.20) to C-5 (δ_C 78.0) and from H-10 (δ_H 1.21) to C-9 (δ_C 76.6). Besides, the chemical shifts of C-5, C-6 (δ_C 91.0) and C-9 revealed the presence of oxygenated carbons. Meanwhile, the chemical shift of C-3 (δ_C 72.1) and the HMBC correlation between H-1′ (δ_H 4.39) and C-3 revealed the attachment of the D-glucopyranoside unit to the position. The NMR data of compound **1** were highly similar to those of scorospiroside, a megastigmane monoglycoside from *Scorodocarpus borneensis* [14] except for the existence of a rutinoside moiety instead of a glucose unit. The relative configuration of compound **1** was determined by a NOESY experiment (Figure 2). The steric relation of the two rings and the orientation of H-3, H-9, H-10 and H-13 were identified by the NOE correlations between H-3 and H-12, H-10 and H-11, H-9 and H-13 as well as H-7 and H-13. These data were identical to those of the (3*S**,5*R**,6*R**,9*R**)-aglycone of scorospiroside and anisoposide B [14, 15]. Thus, the relative configuration of the aglycone could be the same as those structures, and compound **1** was newly identified as *(*3*S**,5*R**,6*R**,9*R**)-6,9-epoxy-3,5-megastigmanediol 3-*O*-rutinoside.

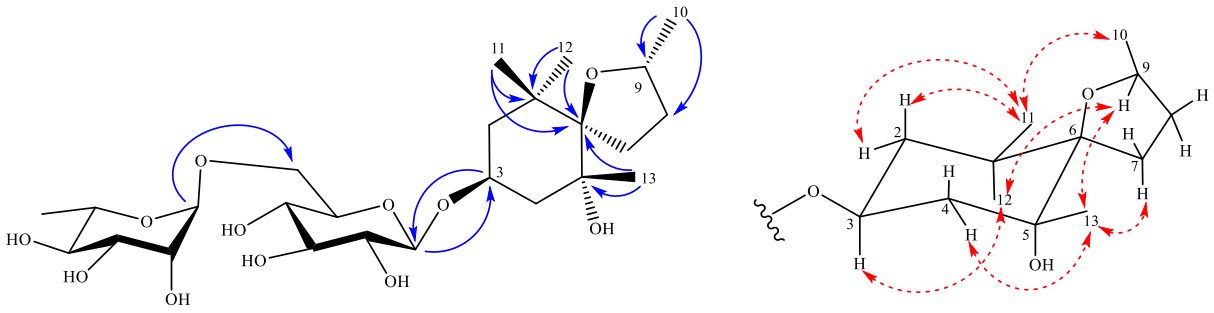

Figure 2. Key HMBC ⟶ and NOESY ⇢ correlations of compound **1**

Structures of 1,7-bis(4-hydroxyphenyl)-5-hydroxy-6-hepten-3-one **(2)**, 3,5,7,4′-tetramethoxyflavone **(3)**, 5,7-dimethoxyflavone **(4)**, indole-3-carboxaldehyde **(5)** and vanillic acid **(6)** were elucidated by comparing their NMR data to those of previous studies [16-20].

The inhibitory effect of the isolated compounds on NO production was evaluated in LPS-induced RAW 264.7 cells. Previously, we reported that the methanol extracts of *A. muricarpum* exhibited a strong NO production inhibition (IC_{50} = 12.67 µg/mL). In addition to NO production inhibition, the methanol extracts were also found to attenuate the expression of iNOS and COX-2 in a concentration-dependent manner [9]. In this study, the diarylheptanoid (**2**) showed a weak effect (IC_{50} = 92.4 µM), while other compounds were inactive up to 100 µM. Intriguingly, the 5,7-dimethoxyflavone **(4)** was the most potent compound with the IC_{50} = 29.5 µM. Similarly, a previous study showed that compound **4** isolated from *Kaempferia parviflora* inhibited the NO production in LPS-induced RAW 264.7 cells at a similar dose [21]. Kim and Hwang also indicated that this compound diminished inflammatory response *via* decreased cytokine (TNF-α and IL-6) in serum and mRNA levels *in vivo* and exhibited a positive effect on the sarcopenic muscle [22].

From the aerial parts of *Amomum muricarpum* Elmer, six compounds were isolated, including a new megastigmane glycoside and five known compounds. This is the first time those compounds were reported from the plant. The inflammatory effects *via* the inhibition of NO production by the isolated compounds were evaluated. Compound 5,7-dimethoxyflavone **(4)** showed significant anti-inflammatory effects *via* inhibition of NO production, while the others exhibited weak inhibition. These results indicated that *Amomum muricarpum* could be a promising target for further biochemical investigations.

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A megastigmane glycoside

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Supporting Information

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