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Natural Product Research
Formerly Natural Product Letters

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/gnpl20

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To cite this article: PCong Pham Van, Hieu Ngo Van, Minh Bui Quang, Nam Duong Thanh, Dan Nguyen Van, Tuan Do Thanh, Ngoc Tran Minh, Hien Nguyen Thi Thu, Trung Nguyen Quang, Thi Thao Do, Loan Pham Thanh, Hien Do Thi Thu & Anh Hoang Le Tuan (2023): Stigmastane-type steroid saponins from the leaves of *Vernonia amygdalina* and their α-glucosidase and xanthine oxidase inhibitory activities, Natural Product Research, DOI: 10.1080/14786419.2023.2188589

To link to this article: https://doi.org/10.1080/14786419.2023.2188589

NATURAL PRODUCT RESEARCH
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Stigmastane-type steroid saponins from the leaves of *Vernonia amygdalina* and their *α*-glucosidase and xanthine oxidase inhibitory activities

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ABSTRACT

Two new vernonioside K (**1**) and vernonioside L (**2**) and four known $\Delta^{7,9(11)}$ stigmastane-type steroidal saponins—vernonioside B2 (**3**), vernoniacum B (**4**), vernonioside B1 (**5**), and vernoamyoside A (**6**)—were isolated from the leaves of *Vernonia amygdalina*. Their structures were determined by comprehensive spectroscopic analysis with one-dimensional nuclear magnetic resonance, two-dimensional nuclear magnetic resonance, and high-resolution mass spectrometry. All isolated compounds (**1**–**6**) were evaluated to determine their inhibitory effects on *α*-glucosidase and xanthine oxidase. Among them, two new compounds **1** and **2** showed significant inhibition of *α*-glucosidase with IC_{50} values of 78.56 ± 7.28 and 14.74 ± 1.57 (μM), respectively, comparable with acarbose as a positive control (127.53 ± 1.73 μM); none of these compounds inhibited xanthine oxidase activity. Compounds **1** and **2** are promising candidates for the development of antidiabetic agents from natural sources.

ARTICLE HISTORY
Received 3 November 2022
Accepted 2 March 2023

KEYWORDS
Vernonia amygdalina; $\Delta^{7,9(11)}$stigmastane-type steroidal saponin; *α*-glucosidase; xanthine oxidase

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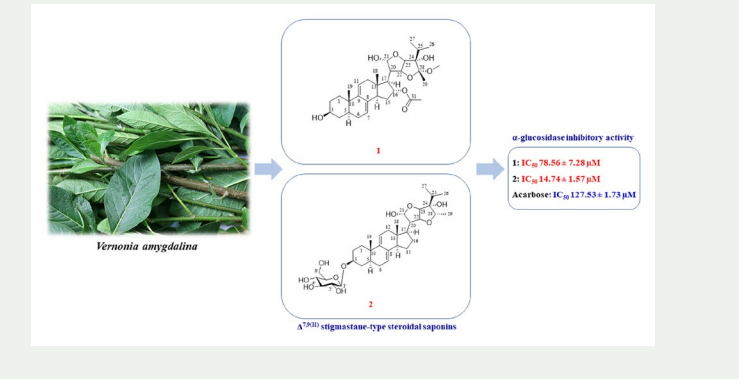
Supplemental data for this article can be accessed online at https://doi.org/10.1080/14786419.2023.2188589.



1. Introduction

Vernonia amygdalina Del. (*Asteraceae*), also known as 'bitter leaf,' is a small shrub with dark green leaves and rough bark that is native to Africa. The leaves of this plant have been regarded as leafy vegetables and used in traditional medicine for the treatment of tonsillitis, fever, malaria, diabetes, pneumonia, jaundice, anemia, stomach problems, and ascariasis (Oyeyemi et al. 2018). *V. amygdalina* is a rich source of secondary metabolites, such as terpenoids, steroids, coumarins, flavonoids, phenolic acids, lignans, saponins, sesquiterpene lactones, tannins, and anthraquinones (Izevbigie 2003; Tona et al. 2004; Muraina et al. 2010; Oyeyemi et al. 2018; Okoduwa et al. 2021; Zhao et al. 2021). Among these, the stigmastane-type steroids are considered characteristic active components of *V. amygdalina*; they have a wide range of biological activities, including anti-inflammatory (Quasie et al. 2016; Nguyen et al. 2021), antidiabetic (Okon and Umoren 2017, Anh et al. 2021), antimalarial (Masaba 2000), and anti-tumor effects (Wong et al. 2013). Here, we describe the identification of six compounds, including two new compounds, from the leaves of *V. amygdalina*, along with their inhibitory activities against *α*-glucosidase and xanthine oxidase.

2. Results and discussion

The methanol extract of the dried leaves of *V. amygdalina* was sequentially partitioned with *n*-hexane and ethyl acetate (EtOAc). Using various chromatographic techniques (silica gel, RP-18 gel, Sephadex LH-20 chromatography columns), two new (**1** and **2**) and four known stigmastane-type steroids (**3**–**6**) were isolated from the EtOAc extract (Figure S1). The chemical structures of the known compounds were identified as vernonioside B2 (**3**) (Rahman et al. 1990; Jisaka et al. 1993; Anh et al. 2021), vernoniacum B (**4**) (Ma et al. 2016; Anh et al. 2021), vernonioside B1 (**5**) (Jisaka et al. 1992; Anh et al. 2021), and vernoamyoside A (**6**) (Quasie et al. 2016), according to the literature.

Compound **1** was obtained as a white amorphous solid. Electrospray ionization high-resolution time-of-flight mass spectrometry (ESI-HR TOF-MS) revealed a quasi-molecular ion peak at *m/z* 583.3248 $[M+Na]^+$ (calcd. for $C_{32}H_{48}NaO_8^+$, 583.3241), suggesting a molecular formula of $C_{32}H_{48}O_8$ (Figure S9, Supplementary Data). The ^1H

and ^{13}C nuclear magnetic resonance (NMR) data of **1** showed the characteristic signals of $\Delta^{7,9(11)}$ stigmastane-type steroidal skeleton (Table S1, Supplementary Data). The ^{1}H NMR spectrum of **1** revealed the presence of two olefinic protons [δ_H 5.33 (1H, brs, H-7), 5.44 (1H, d, J = 6.0 Hz, H-11)], a distinctive H-3 multiplet [δ_H 3.78 (1H, m, H-2)], an isopropyl group [δ_H 1.11 (3H, d, J = 6.6 Hz, H-26), 1.23 (3H, d, J = 6.6 Hz, H-27)], two angular methyl protons [δ_H 0.61 (3H, s, H-18), 0.86 (3H, s, H-19)], another methyl proton [δ_H 1.59 (3H, s, H-29)], an acetylate methyl group [δ_H 2.21 (3H, s, 16-OAc)], and a methoxyl group [δ_H 3.29 (3H, s, 28-OCH_3)]. ^{13}C NMR showed 29 carbon signals, including six quaternary carbons, six methylene groups, four olefinic carbons, an acetyl group, five methyl groups, and a methoxy group. The planar structure of **1** was further supported by the heteronuclear multiple bond correlation (HMBC) spectrum (Figure S2, Supplementary Data). The HMBC correlations between H-11 and C-8/C-9/C-10/C-13 and between H-7 and C-5/C-8/C-9/C-15 indicated that the two double bonds were at 7(8) and 9(11) positions. The HMBC cross-peaks from H-16 to δ_C 170.8 (CH_3COO) indicated that the acetyl group was located at C-16. For the side chain, the HMBC spectrum showed the connection of H-26/H-27 and C-24, suggesting that the isopropyl moiety was attached to C-24. Additionally, the positions of methoxy and methyl groups at C-28 were deduced by the HMBC correlation of 28-OCH_3/H-29 with C-28. The HMBC cross-peaks from H-20 to C-21/C-22, from H-21 to C-22/C-23, from H-22 to C-20/C-23, and from H-23 to C-21/C-24 confirmed the presence of two furan rings, which were connected *via* C-22 and C-23. Finally, the side chain was attached to C-17 by the HMBC correlation of H-17 and C-20. The nuclear Overhauser effect spectroscopy (NOESY) correlations between H-3 and H-5, H-14 and H-17, and H-18 and H-16 and H-19, indicated that rings A/B and C/D fused *in trans*; H-16, H-18, and H-19 were in the β configuration; and H-3, H-5, and H-17 were in the α configuration. Furthermore, the NOESY correlation between H-17 and 28-OCH_3 indicated that these protons adopted the α configuration, whereas correlations between H-20 and H-18/H-21/H-27, between H-23 and H-22/H-27/H-29 indicated that these protons were in the β configuration (Figure S2, Supplementary Data). Therefore, the stereochemistry of the side chain was determined as shown in Figure S1. Finally, the structure of compound **1** was elucidated and named vernonioside K.

Compound **2** was isolated as a white amorphous solid. The molecular formula ($C_{35}H_{54}O_{10}$) was determined by ESI-HR TOF-MS with a chlorinated molecular ion peak at *m/z* 669.3402 $[M+Cl]^-$ (calcd. for $C_{35}H_{54}ClO_{10}^-$, 669.3411) (Figure S16, Supplementary Data). The ^{1}H NMR spectrum of **2** showed signals of two angular methyl groups [δ_H 0.65 (3H, s, H-18), 0.88 (3H, s, H-19)], an isopropyl group [δ_H 1.13 (3H, d, J = 6.7 Hz, H-26), 1.23 (3H, d, J = 6.7 Hz, H-27)], another methyl group [δ_H 1.25 (3H, d, J = 6.8 Hz, H-29)], and two olefinic protons [δ_H 5.41 (1H, s, H-7), 5.22 (1H, s, H-11)] (Table S1). Furthermore, an anomeric proton of the glucopyranosyl unit [δ_H 5.02 (1H, d, J = 7.7 Hz, H-1′)] was observed in the ^{1}H NMR spectrum. The β configuration of this proton was determined by the large $J_{1',2'}$ value coupling constant (J = 7.7 Hz) (Table S1); the absolute configuration was deduced as β-d-glucopyranoside by acid hydrolysis and comparison of the R_f with authentic d-glucose (TLC, MC: MeOH:H_2O = 8:5:1, R_f = 0.3) (Vu et al. 2021). The HMBC correlation from δ_H 5.02 to δ_C 77.3 showed that the sugar moiety was connected to C-3 of aglycone. The ^{13}C NMR spectrum of **2** exhibited 35 carbon signals, of which 29 and 6 signals were ascribable to the steroidal aglycone

and to β-d-glucose, respectively. The ^{1}H and ^{13}C NMR data of **2** revealed the typical $\Delta^{7,9(11)}$ stigmastane-type steroidal skeleton (Zhao et al. 2021) and were similar to **3** except for the absence of a hydroxy group at C-16 and a methoxy group at C-28, based on the obvious upfield shifts of C-16 (δ_C 27.7) and C-28 (δ_C 84.7) in **2** compared with C-16 (δ_C 76.2) and C-28 (δ_C 113.4) in **3** [5, 6]. The NOESY spectrum of **2** showed correlations from H-3 to H-5, from H-14 to H-17, and from H-18 to H-19 and H-20 suggesting the *trans* fusion of the rings A/B and C/D; α-orientation of H-3, H-5, H-14, and H-17; and β-orientation of H-18, H-19, and H-20. The NOESY cross-peaks from H-20 to H-18/H-21, from H-21 to H-18/H-26, and from H-26 to H-22/H-23/H-28 indicated that these protons were in the β configuration. The NOESY correlation between H-17 and H-14/H-29 showed that H-29 was in the α configuration. Therefore, the structure of compound **2** was elucidated as shown in Figure S1 and named vernonioside L.

α-Glucosidase is used in the treatment of type 2 diabetes mellitus (Kumar et al. 2011, Ha et al. 2018). α-Glucosidase inhibitors act by competitive inhibition of α-glucosidase at the brush borders of the intestinal epithelium. As a result, the digestion of complex carbohydrates is delayed and the absorption of glucose is shifted, thereby allowing the sluggish insulin secretion to 'catch up' with carbohydrate absorption (Hossain and Pervin 2018). In this study, the anti-α-glucosidase activities of compounds **1**–**6** were evaluated, using acarbose as a positive control. Among the compounds tested, the two new compounds **1** and **2** showed significant inhibitory effects on α-glucosidase with IC_{50} values of 78.56 ± 7.28 and 14.74 ± 1.57 μM, respectively, which were stronger than the positive control (acarbose 127.53 ± 1.73 μM). These observations showed that compounds **1** and **2** have the potential for use in treatments for type 2 diabetes mellitus *via* the inhibition of α-glucosidase activity. However, further *in vivo* studies and clinical trials are required.

Xanthine oxidase is a key enzyme that catalyzes the last step in the conversion of purines to uric acid; it plays a vital role in the development of hyperuricemia and gout (Lund 2010). Allopurinol is a xanthine oxidase inhibitor prescribed for the treatment of gout. Therefore, the inhibitory effects of compounds **1**–**6** against xanthine oxidase activity were evaluated. As shown in Table S2, none of these compounds significantly inhibited xanthine oxidase activity.

2.1. Extraction and isolation

The dried leaves of *V. amygdalina* (1.5 kg) were extracted with methanol (MeOH) (9 *L* × 3 times × 2.5 h) under sonication at 45 °C. The combined extracts were filtered and evaporated under reduced pressure to yield a brown residue (100.0 g). This extract was suspended in hot distilled water and successively partitioned with *n*-hexane and ethyl acetate, yielding *n*-hexane (30.0 g), ethyl acetate (EtOAc, 45.0 g), and water (W, 20.0 g) fractions. The EtOAc fraction was subsequently chromatographed on a silica gel column and eluted with a gradient mixture of dichloromethane (CH_2Cl_2):MeOH (99/1 → 0/100, v/v) to yield six fractions (EA1–EA6). Fraction EA2 (5.2 g) was then purified on an RP-18 gel column and eluted with MeOH:H_2O (2.5/1, v/v), yielding compounds **1** (5.0 mg) and **5** (6.0 mg). Fraction EA3 (15.0 g) was separated on a silica gel column using a solvent gradient of CH_2Cl_2:MeOH (99/1 → 0/100, v/v) to obtain four fractions (EA3A–EA3D). Compound **3** (5.1 mg) and three

fractions (EA4A–EA4C) were obtained from fraction EA3B (5.3 g) using an RP-18 column with a solvent system of MeOH:H_2O (3/1, v/v). Fraction EA4B (0.5 g) was further purified on a Sephadex LH-20 column and eluted with MeOH to produce compound **4** (8.0 mg). The fraction EA5 (12.0 g) was applied to a Sephadex LH-20 column with an isocratic mixture of MeOH:H_2O (2/1, v/v) yielding three subfractions (EA5A–EA5C). The fraction EA5B (3.5 g) was passed through an RP-18 gel column with a mixture of MeOH:H_2O (2:1, v/v), which yielded compound **2** (3.0 mg) and compound **6** (6.5 mg).

3. Conclusion

Two new $\Delta^{7,9(11)}$ stigmastane-type steroidal saponins, vernonioside K and L, together with four known compounds: vernonioside B2 (**3**), vernoniacum B (**4**), vernonioside B1 (**5**), and vernoamyoside A (**6**) were isolated from the leaves of *V. amygdalina*. The structures of the new compounds were elucidated from one-dimensional NMR, two-dimensional NMR, and MS data. Compounds **1**–**6** were examined for inhibitory effects on *α*-glucosidase and xanthine oxidase. Novel compounds **1** and **2** exhibited significant anti-*α*-glucosidase activity with IC_{50} values of 78.56 ± 7.28 and 14.74 ± 1.57 μM, respectively, which were comparable with the positive control (acarbose, 127.53 ± 1.73 μM). However, none of the examined compounds showed inhibition of xanthine oxidase. This study provided phytochemical evidence for further development of new anti-*α*-glucosidase lead compounds, along with investigations of their chemical modifications and mechanisms of action.

Disclosure statement

The authors declare that they have no conflict of interest.

Funding

This research was supported by Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 104.01-2020.11. In addition, Pham Van Cong was funded by Vingroup Joint Stock Company and supported by the Domestic Master/Ph.D. Scholarship Programme of Vingroup Innovation Foundation (VINIF), Vingroup Big Data Institute (VINBIGDATA), code VINIF.2022.TS017.

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