New steroidal saponin from *Antigonon leptopus* Hook. and *Arn.*

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ABSTRACT

Background: Antigonon leptopus Hook. and Arn., Polygonaceae (cadena de amor), is a herbal remedy for pain and gout-like symptoms in the Philippines. The methanol extract of A. leptopus have shown strong inhibitory action against xanthine oxidase. **Objective:** To isolate and identify the compound responsible for the xanthine oxidase inhibitory action. **Materials and Methods:** A bioassay-guided isolation scheme using an in vitro assay for the inhibition of xanthine oxidase was employed. The structure was established using spectroscopic analysis and chemical methods. **Results:** The isolated compound was determined to be a noncompetitive inhibitor of xanthine with an IC50 of 1.79 μg/mL. **Conclusion:** The isolated compound may represent a new class of xanthine oxidase inhibitors.

Key words: Gout, medicinal plants, saponin, uric acid, xanthine oxidase

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INTRODUCTION

Antigonon leptopus Hook. and Arn. is a woody, perennial member of the buckwheat (*Polygonaceae*) family commonly found in tropical Asia, Africa, the Caribbean and the Americas. Its common names include cadena de amor, flores kadena, bride's tears, chain-of-love and confederate vine. It is propagated by seeds or cuttings and is mostly used as an ornament. The bark, fruit, leaves, and seeds of this plant also have widespread applications in folkloric medicine. In the Philippines, the aerial parts of the plant are used as an anti-inflammatory agent and for wound healing.[1,2] In Trinidad and Tobago, the leaves are used for diabetes, urinary problems and low blood pressure.[3] Preliminary studies on the activity of crude extracts of *A. leptopus* in inhibiting the action of xanthine oxidase (XO) had been done. Results of phytochemical profiling included cardiac glycosides, steroids, tanins and terpenoids among possible types of compound responsible for this bioactivity.[4] Being a traditional medication used for inflammation and pain, these compounds may potentially find application as new XO inhibitors.

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The only commercially available XO inhibitor to date is allopurinol (1,5-dihydro-4H-pyrazolo[3,4-d] pyrimidin-4-one), a purine analog in clinical use for >30 years.[5] Despite generally acceptable efficacy and safety profiles, very rare but serious adverse reactions of allopurinol administration occur including interstitial nephritis, renal failure, hepatotoxicity, vasculitis, and an array of skin rashes varying from mild to very severe and life-threatening allopurinol hypersensitivity syndrome.[6,7] The recurrence and severity of gout has been reportedly increasing over the last decades and it continues to be a major health problem due to shifts in diets and lifestyles.[8]

As part of our effort to search for new biologically active compounds from local herbal remedies, a new saponin, with a steroidal backbone was isolated from *A. leptopus*. In this paper, we present the result of the isolation, structure elucidation and XO inhibitory activity of this new compound.

MATERIALS AND METHODS

Materials and instruments

Absorbance measurements were done using Thermo Scientific Multiskan Go®. Shimadzu LC-10 high performance liquid chromatography (HPLC) system with ultraviolet (UV)-visible detector and Phenomenex semi-prep reverse phase column (00G-4461-E0) were used in HPLC analyses. Infrared spectra were obtained using a Shimadzu Fourier transform infrared (FTIR)

Prestige® while melting point was determined using Fischer-Johns melting point apparatus. Mass spectral data were obtained using 5800 AB-SCIEX matrix assisted laser desorption ionization-collision induced dissociation-time-of-flight (MALDI-CID-TOF/TOF). ^{1}H, ^{13}C and two-dimensional-NMR data were obtained using a Bruker Avance III 600 Ultrashield Plus (600 MHz). All reagents used were purchased and used as per instruction regarding handling and storage, including distilled technical grade methanol (MeOH), ethyl acetate (EtOAc) and hexane (Hex) used for crude extraction, XO from bovine milk (Sigma X4500), xanthine (Sigma X7375), allopurinol (Sigma A8003), KH_2PO_4 (Sigma P3786), Na_2HPO_4 (Sigma S7907) and DMSO (Sigma D1435) for the inhibitory assay, LH-20 (Sephadex), silica gel (Merck, 45 um mesh), analytical grade MeOH (JT Baker), HPLC grade acetonitrile (ACN) (JT Baker) and HPLC grade water (H_2O) (JT baker) for isolation and purification.

Plant material

Antigonon leptopus leaves were collected from various sites around the University of the Philippines-Diliman campus from the period of June 2010 to June 2011. Representative plant samples were submitted to the Institute of Biology Herbarium for proper identification and authentication. Existing voucher specimen 1809 was matched to the collected samples.

Crude extraction

Mature leaves of *A. leptopus* were selected and washed with running tap water. Samples were air-dried atop acid-free paper for 3 days. The dried samples were weighed and homogenized prior to exhaustive extraction with distilled technical grade MeOH. The extracts were filtered and concentrated *in vacuo* yielding the crude methanolic extract. The methanolic extract was partitioned between hexane and distilled deionized water (6:1). Solvent partitioning was repeated until an almost colorless hexane layer was collected and concentrated *in vacuo*. Subsequent extraction of the aqueous layer with EtOAc (6:1) was done and repeated until an almost colorless organic layer was collected. The aqueous extract was freeze dried.

Column chromatography of bioactive fractions

An enzyme-guided isolation and purification scheme was used to isolate the active compounds and was done as follows: The dry weight of the active fraction was recorded and a minimum amount of MeOH was added and mixed thoroughly until homogenous prior to loading over the prepared column. A total of 2 g of aqueous extracts was used per run.

A total of 50 g of Sephadex LH-20 was swelled in analytical grade MeOH for 24 h prior to loading (2 g). A glass column with diameter of 3 cm was packed up to a height of 60 cm. Isocratic elution with MeOH was performed. Eluates were collected per 5 mL volume in 20 mL scintillation vials. Column was regenerated by thoroughly washing with MeOH after elution of each batch of extracts. Eluates were dried *in vacuo* using a Senti-Vac™ system. UV-visible profiles of all eluates were determined from 200 to 700 nm using MeOH as solvent. Fractions with similar profiles and maximum absorption were pooled.

Isolates were further purified using reversed phase HPLC (RP-HPLC), gradient elution with H_2O and ACN. Fractions with the same retention times were collected. Peaks at 5, 20, 34 and 43 min were collected and pooled. Optimized parameters are as follows: λ_{Abs} = 254 nm; total flow rate: 2 mL/min; gradient: 0.01 min = 5% ACN, 5.01 min = 5% ACN, 11.01 min = 11% ACN, 60.01 min = 50% ACN, 75.01 min = 100% ACN, 80.01 min = 5% ACN, 85.01 min = 5% can.

Characterization

Uncorrected melting point was determined. For FTIR analyses, samples were prepared by spread-plate technique using KBr plates. UV-visible spectra and maximum absorption of all fractions were obtained by dissolving in MeOH and recording absorbance from 200 to 700 nm. Mass spectra were obtained using a MALDI-CID-TOF/TOF machine operated with laser intensity set at 4100-4300, using 2,5-dihydroxybenzoic acid as matrix.

^{1}H and ^{13}C-NMR, distortion less enhancement by polarization transfer (DEPT), hetero nuclear multiple quantum correlation (HMQC), heteronuclear multiple-bond correlation spectroscopy (HMBC), double quantum filtered-correlated spectroscopy (DFQ-COSY), total correlation spectroscopy (TOCSY), and nuclear over hauser effect spectroscopy (NOESY) were all obtained using a 600 MHz NMR with D_2O as solvent. NMR spectra were analyzed using MestReNova 7 by Mestrelab Research

Compound AF0501, $C_{38}H_{62}O_{14}$. White amorphous powder, soluble in water. RP-HPLC retention time: 43. 1 min MP: 168°C. IR: ~3600-3000/cm (broad strong), 2900-2800/cm, 1300.00/cm, 1700 (strong)/cm. MALDI-CID-TOF/TOF: m/z 743.6430. ^{1}H NMR (600 MHz, D_2O): δ 4.14 (dd, J = 7.7, 6.3 Hz, 1H), 4.07 (dt, J = 4.9, 2.6 Hz, 1H), 4.00 (m, 1H), 3.99 (m, 1H), 3.91 (m, 1H), 3.85 (bd, 1H), 3.76 (dt, J = 4.7, 2.7 Hz, 1H), 3.68 (dd, J = 12.86, 3.23 Hz, 1H), 3.66 (dd, J = 9.8, 4 Hz, 1H), 3.60 (dd, J = 7.3, 3.2 Hz, 1H), 3.53 (dd, J = 4.5.3, 2.3 Hz, 3H), 3.29 (s, 1H), 3.27 (d, J = 3.2 Hz overlap, 1H), 3.06 (td, J = 13.51, 13.43, 3.20 Hz, 1H), 2.52 (dd, J = 13.31, 3.2 Hz, 2H), 2.35 (m, 1H), 2.26 (dd, J = 13.5, 2.3 Hz, 1H), 2.24 (d, J = 7.5 Hz, 1H), 2.17 (ddd,

J = 13.67, 4.80, 2.55 Hz, 3H), 2.11 (m, 2H), 2.00 (m, 2H), 1.95 (m, 1H), 1.68–1.65 (m, 7H), 1.50–1.35 (m, 3H), 1.34 (d, J = 6.8 Hz, 2H), 1.29 (t, J = 7.3 Hz, 1H), 1.05 (d, J = 6.99, 6H), 1.00 (d, J = 7.0 Hz, 3H).

^{13}C NMR (151 MHz, D_2O) δ 173.37, 136.75, 124.09, 100.01, 98.69, 92.28, 80.4, 76.1, 71.19, 67.39, 67.37, 67.35, 66.97, 65.51, 62.44, 62.37, 59.41, 58.98, 57.84, 46.02, 43.61, 41.4, 39.25, 38.71, 35.84, 34.67, 32.72, 29.89, 29.03, 28.93, 26.47, 23.54, 22.53, 21.77, 20, 17.9, 16.57.

Xanthine oxidase inhibition assay

The XO inhibitory activity was assessed spectrophotometrically using a 96-well plate reader under aerobic conditions. The assay mixture consisted of the *A. leptopus* extract dissolved in 1% DMSO, 0.15 M phosphate buffer at pH 7.5 and the XO enzyme solution at 0.25 U/mL. All solutions were prepared just before use. After preincubation at 25°C for 10 min, the reaction was initiated by adding 0.6 mM of xanthine dissolved in phosphate buffer. Absorption increments were monitored every 30 s for 10 min at 295 nm to monitor the rate of formation of uric acid. Allopurinol was used as a positive control and XO inhibitory activity was expressed as percentage inhibition.

RESULT AND DISCUSSION

The pure compound was obtained as white amorphous powder with λ_{max} at 220 nm, characteristic of an isolated C = C bond. Uncorrected melting point was determined to be 168°C. The infrared spectrum was obtained and observed peaks were assigned to corresponding functional groups. Molecular formula was determined to be $C_{38}H_{62}O_{14}$ (m/z 743.6430, $[M + H]^+$) by positive ion MALDI-CID-TOF/TOF. Fragmentation pattern showed major peaks at 663.6703, 602.3524, 522.3897, 442.4178 (base peak), 303.1420 and 221.1722.

Direct correlations (^{1}J) were established using HMQC. Related spin systems and long range homonuclear bond correlations were established using data extracted from DFQ-COSY (^{2}J-^{5}J) and TOCSY (^{2}J-^{5}J) spectra. Heteronuclear (C-H) correlations, from ^{2}J to ^{5}J, were verified using HMBC. Spatial correlations were established using data from the NOESY spectrum.

The ^{13}C-NMR spectrum gave 37 carbon signals and analysis of the DEPT spectra (DEPT 90 and DEPT 135) showed three quaternary carbons, twenty-two methines, four methyl and eight methylene carbons. Signal at $C_{173.37}$ indicates an acyl carbon with olefinic carbons at $C_{136.75}$ and $C_{124.09}$. Several carbon atoms connected to oxygen(s) were observed with chemical shifts ranging between 60 ppm and 100 ppm. The presence of the two methyl groups at $C_{17.9}$ and six hydrogens at around 1.05-1.00 ppm, supported by the two-dimensional-NMR correlations, suggested that the isolated compound indeed has 38 carbons.

The ^{1}H NMR displayed two anomeric signals at $H_{4.14}$ and $H_{4.07}$ with corresponding carbons at $C_{100.01}$ and $C_{98.67}$, respectively. These, together with the ^{1}H shifts connected to carbons between 60 and 100 ppm suggests that the compound has two sugar moieties. The monosaccharide units were identified as rhamnopyranose by correlations derived from TOCSY to COSY correlations and was further supported by the presence of the methyl groups as doublets at $C_{17.9}$-$H_{1.05}$ (J = 6.99 Hz). Comparison with reported literature values[9-14] and the data obtained from the ^{1}H NMR coupling constant for the anomeric proton between 4 and 8 Hz are consistent with the β-configuration for L-rhamnose.[9-13] In contrast, the coupling constants for the α-configuration of L-rhamnose is <3.0 Hz.[15-18] Hence, the rhamnopyranoside units of the isolated compound were determined to be in β-configuration.

Glycosidic linkage to the corresponding two rhamnopyranosyl moieties was established at $C_{65.57}$ and is esterified at $C_{173.37}$. For the first spin system, HMBC correlations observed between $C_{173.37}$ and $H_{4.01}$ (^{3}J) and $H_{2.0}$ (^{4}J) confirming the connectivities. For the aglycone backbone, extensive, and long range H-H correlations were identified based from TOCSY and the key HMBC correlations are as follows: $H_{1.0}$-$C_{34.67}$ (^{3}J), $H_{1.0}$-$C_{59.41}$ (^{4}J), $H_{1.34}$-$C_{59.41}$ (^{3}J), $H_{1.29}$-$C_{34.67}$ ($_3$J), $H_{3.29}$-$C_{38.71}$ (^{3}J), $H_{1.62}$-$C_{62.37}$ (^{3}J), $H_{1.62}$-$C_{41.4}$ (^{4}J) and $H_{1.68}$-$C_{57.84}$ (^{3}J). Figure 2 summarizes the key correlations. It was identified to be steroidal in nature as established by the core seventeen carbon-unit backbone (cyclopentanoperhydrophenanthrene). The β-configuration of the rhamnoside moieties were verified based from absence of NOESY correlation between $H_{3.53}$ and neighboring H's at 3.99 and 3.66 ppm for rhamnopyranose connected at $C_{65.57}$ and $H_{4.0}$ and H's at 3.66 and 3.76 ppm for rhamnopyranose connected at $C_{173.37}$. For the aglycone, NOESY correlations between $H_{1.0}$-$H_{1.29}$, $H_{1.62}$-$H_{2.35}$-$H_{2.24}$ and $H_{3.27}$-$H_{3.68}$ verify the structure. Furthermore, fragments corresponding to the monoisotopic peaks derived from mass spectral data support the fragmentation of this structure. The base peak, m/z 442.4178, corresponds to the monoisotopic peak of the aglycone. The structure of the steroidal saponin is shown in Figure 1.

Saponins and related triterpenoids and steroids had been the focus of recent studies due to their potential efficacies. They had been widely used in many countries as traditional medicine. The most popular class of these compounds is the ginsenosides mainly found in *Panax ginseng*. Used in traditional Asian medicine, many studies had focused on the isolation,

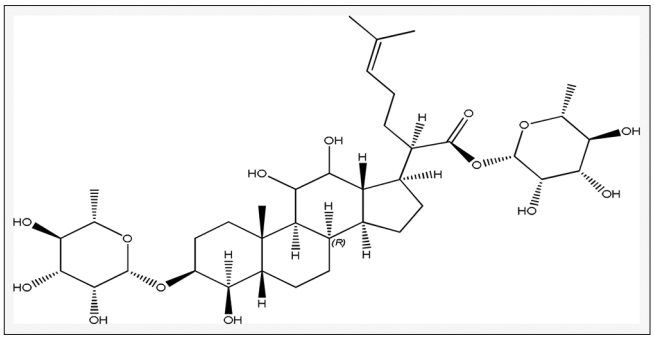

Figure 1: Structure of new steroidal saponin with xanthine oxidase inhibitory activity isolated from Antigonon leptopus

structure elucidation and bioactivity of ginsenosides. Most notably studied are the activities of ginsenosides as antioxidants and as sources of anti-cancer compounds.[19]

The inhibitory activities of the crude MeOH, EtOAc, hexane and aqueous extracts of *A. leptopus* were determined. It has been reported previously that extracts having >50% enzyme inhibition at 50 μg/mL warranted further investigation.[20] Of the four, the aqueous extract inhibited XO at this level as demonstrated by a percent inhibition of 52.19% at 33.3 μg/mL. A significant loss of inhibitory activity was observed after solvent partitioning of the methanolic extract with EtOAc and Hex with percent inhibitions at 21.86% and 22.41%, respectively at 33.3 μg/mL. Inhibitory activities of fractions further purified by column chromatography using Sephadex LH-20 were determined. Results show that the activity of the pure compound is 77.45% at the same concentration with IC_{50} at 1.79 μg/mL. For comparison purposes, the IC_{50} value of allopurinol under the same experimental conditions was 1.00 μg/mL.

The calculated experimental K_i is 2.53 ± 0.001 μM. The values of V_{max}, on the other hand, decrease with increasing concentration of AF0501. Experiemental values are indicative of a noncompetitive type of inhibition. The experimental K_i value for the pure compound is comparable to those obtained by other authors who performed similar experiments on possible noncompetitive inhibitors of xanthine oxidase.[21] This value is lower than the previously studied noncompetitive XO inhibitors, 2-alpha-bromo-benzimidazole (K_i = 46 μM) and 2-amino-6-hydroxy-8-mercaptopurine and 2-amino-6-purinethiol (APT) at 5.78 μM and 6.61 μM, respectively.[21] Since K_i is indicative of affinity of the inhibitor to the enzyme, a lower Ki value potentially means that AF0501 may inhibit XO more effectively. Furthermore, since the IC_{50} of AF0501 is comparable to that of allopurinol, the isolated compound may represent a new class of XO inhibitors.

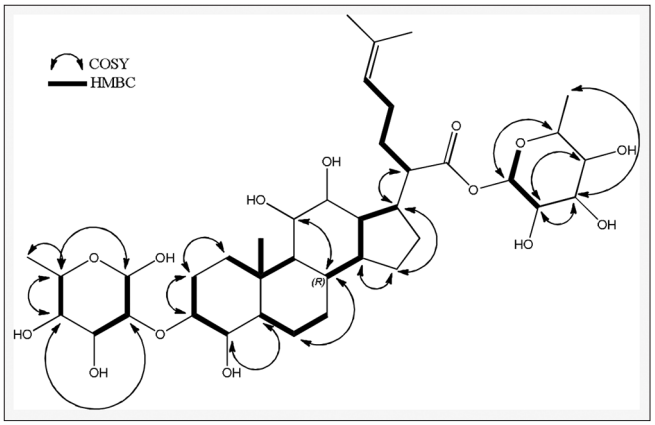

Figure 2: Key correlations for the structure elucidation of the new compound

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