Two New Cytototoxic Cardenolides from the Whole Plants of *Adonis multiflora* Nishikawa & Koki Ito

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Abstract: A phytochemical investigation of the whole plants of *Adonis multiflora* Nishikawa & Koki Ito. resulted in the isolation and identification of two new cardenolides—adonioside A (1) and adonioside B (6)—as well as four known cardenolides: tupichinolide (2) oleantrine (3), cryptostigmin II (4), and cymarin (5). Their structures were elucidated on the basis of NMR, MS, and IR spectroscopic analyses. Compounds 1, 2, 5, and 6 showed significant cytotoxicity against six human cancer cell lines (HCT-116, HepG2, HeLa, SK-OV-3, and SK-MEL-5, and SK-BR-3).

Keywords: *Adonis multiflora*; adonioside A; adonioside B; cardenolide; cytotoxic activity

1. Introduction

Cardenolides, a chemical class within the cardiac glycosides, have a five-membered lactone group in the β position at C17 [1]. The mechanisms of these compounds are known to inhibit Na⁺/K⁺-ATPase, activate the cation pump, and increase in intracellular calcium concentration through cellular output of Na⁺ and intake of K⁺ [2]. Because of these biological actions, cardenolides have been used in the treatment of heart failure [3]. In addition, many researchers have suggested that cardenolides may inhibit the growth of cancer cells, and have described them as anticancer agents with fewer side effects [4,5].

Cardiac glycosides were isolated from several plant families of Ranunculaceae, Scrophulariazea, Apocynaceae, and Liliaceae, along with pregnant glycosides [6]. In Korea, the *Adonis* family is mainly comprised of three species, *A. amurensis*, *A. pseudoamurensis*, and *A. multiflora* based on RAPD analysis [7,8]. Previous phytochemical studies conducted on the roots of *A. amurensis*, the most well-known *Adonis* species, have identified several cardenolides: corchoroside A, coffallatoxin, cymarin, cymarol, digitoxigenin 3-O-β-D-cymaroside, k-strophanthin, and k-strophanthin-β [9]. However, little has been reported concerning the biological and phytochemical properties of *A. multiflora*, except a brief report [10]. We have confirmed the presence of cardenolide spots in the TLC of ethanolic extracts from whole plants of *A. multiflora* based on the UV absorption pattern and the colors produced by spraying with a 10% H₂SO₄ solution and heating. Over the course of investigating cardenolides in whole plants of *A. multiflora* Nishikawa & Koki Ito, two new
cardenolides 1 and 6 were identified and structurally determined, along with four known ones 2–5 (Figure 1). The cardenolides were then evaluated for cytotoxicity against six human cancer cell lines (HCT-116, HepG2, HeLa, SK-OV-3, SK-BR-3, and SK-MEL-5).

![Figure 1. Compounds 1–6 isolated from the whole plants of Adonis multiflora.](image)

2. Results and Discussion

The EtOH extracts were partitioned into CH₂Cl₂, EtOAc, BuOH, and H₂O fractions. Repeated SiO₂ and ODS column chromatography of the CH₂Cl₂ and BuOH fractions resulted in the identification of two new cardenolides, named adonioside A (1) and adonioside B (6), along with four known cardenolides 2–5. The known compounds were identified as tupichinolide (2), oleandrine (3), cryptostigmin II (4), and cymarin (5) on the basis of spectroscopic analysis and the identities were confirmed by comparing their measured spectroscopic data with those reported in the literature [11–14].

Compound 1 was isolated as a white powder and showed IR absorbance bands representing OH (3384 cm⁻¹), CHO (1737 cm⁻¹), and C=O (1639 cm⁻¹) groups. The molecular weight was determined to be 606 from the molecular ion peak m/z 605 [M − H]⁻ in the negative FAB-MS spectrum, and a molecular formula of C₃₂H₄₅O₁₁ was determined from the high-resolved molecular ion peak ([M − H]⁻, m/z 605.2971, calc. for C₃₂H₄₅O₁₁, 605.2962) in the negative HR-FAB-MS. The ¹H-NMR spectrum (Table 1) exhibited the characteristics of an α,β-unsaturated-γ-lactone ring, with signals at δ(H) 5.95 (dd, J = 1.6, 1.6 Hz, H-22), 4.94 (dd, J = 18.4, 1.6 Hz, H-21a), and 4.83 (dd, J = 18.4, 1.6 Hz, H-21b) as well as a tertiary methyl signal at δ(H) 0.92 (s, H-18), a formyl signal at δ(H) 9.92 (s, H-19), two O-bearing CH signals at δ(H) 4.19 (br.s, H-3) and 5.41 (ddd, J = 9.6, 8.4, 8.4 Hz, H-16), and an AcO signal at δ(H) 1.95 (s, H-AcO), which suggested the presence of a cardenolide moiety with two
oxygenated methines and an AcO group. In addition, a hemiacetal signal at δ(H) 4.50 (dd, J = 9.6, 2.4 Hz, H-1'), three O-bearing CH signals at δ(H) 3.31~3.67 (H-3'~5'), an O-bearing CH3 signal at δ(H) 3.36 (s, H-CH3O), a CH2 signal at δ(H) 2.05 (m, H-2'), and 1.66 (m, H-2''), and a CH3 signal at δ(H) 1.33 (d, J = 6.8 Hz, H-6'), indicated that 1 was a cardiac monoglycoside with a β-digonopyranoside.

The 13C-NMR spectrum showed 32 C-atoms signals (Table 1). The aglycone with α,β-unsaturated-γ-lactone ring signals observed at δ(C) 173.9 (C-23), 167.3 (C-20), 121.5 (C-22), and 75.5 (C-21), a formyl signal at δ(C) 208.1 (C-19), two O-bearing quaternary signals at δ(C) 73.5 (C-5) and 83.9 (C-14), AcO signals at δ(C) 170.4 (C-OAc) and 21.0 (C-OAc), two O-bearing CH signals at δ(C) 74.1 (C-3) and 73.7 (C-16), and a tertiary CH3 signal at δ(C) 15.8 (C-18) indicated that the aglycone was a cardenolide with four hydroxyls, one formyl, and one AcO group. The monosaccharide carbon signals, including a hemiacetal signal at δ(C) 98.9 (C-1'), three O-bearing CH signals at δ(C) 77.6 (C-3'), 70.7 (C-5'), 66.9 C-4', a CH3O signal at δ(C) 55.8 (C-CH3O), a CH2 signal at δ(C) 31.5 (C-2'), and a CH3 signal at δ(C) 16.7 (C-6'), allowed us to conclude that the sugar was β-digonopyranose.

Acid hydrolysis of 1 and purification of the hydrolysate using column chromatography resulted in a sugar compound, which was identified to be a digonopyranose by direct comparison between its "Rf" values on the SiO2 TLC (0.47 with CHCl3/MeOH 9:1, and 0.19 with CH2Cl2/EtOH 9:1) and those of an authentic sample. The specific rotation value of the obtained sugar ([α]D20 = +56.8, c = 0.11, H2O), and the large J value of the anemic signal at δ(H) 4.50 (dd, J = 9.6, 2.4 Hz, H-1') revealed the sugar to be β-D-digonopyranose. The location of β-D-digonopyranose, methyl, formyl, hydroxyls, and AcO groups of 1 were determined from the connectivity between the oxygenated methine proton δ(H) 4.50 (1H, dd, J = 9.6, 2.4 Hz, H-1') and O-bearing CH carbon δ(C) 74.1 (C-3), tertiary methyl proton δ(H) 0.92 (s, H-18) and quaternary carbon δ(C) 49.8 (C-13), formyl proton δ(H) 9.92 (s, H-19) and quaternary carbon δ(C) 54.3 (C-10), tertiary methyl proton δ(H) 0.92 (s, H-18) and O-bearing quaternary carbon δ(C) 83.9 (C-14) and O-bearing methine methine proton δ(H) 5.41 (dd, J = 9.6, 8.4, 8.4 Hz, H-16) and AcO carbon δ(C) 170.4 (C-OAc) in the HMBC spectrum, respectively. The location of the lactone group was deduced from the connectivity between the methylene protons H-15 δ(H) 2.59 (dd, J = 15.6, 9.6 Hz, H-15), O-bearing methine proton δ(H) 5.41 (dd, J = 9.6, 8.4, 8.4 Hz, H-16) and the methine proton δ(H) 3.15 (d, J = 8.4 Hz, H-17) in the COSY spectrum (Figure 2). Taken together, compound 1 was determined to be a 16-β-acetoxythropanthidin 3-O-β-D-digonopyranoside, a new cardenolide named adoniside A.

Compound 6 was also isolated as a white powder and showed IR absorbance bands of OH (3387 cm⁻¹), CHO (1742 cm⁻¹), and C=C (1647 cm⁻¹) groups. The molecular weight was determined to be 710 due to the pseudomolecular ion peak m/z 733 [M + Na]⁺ in the positive FAB-MS spectrum, and the molecular formula of C₃₆H₄₅O₁₄ was determined by the high-resolution pseudomolecular ion peak ([M + Na]⁺, m/z 733.3511, calc. for C₃₆H₄₅O₁₄Na, 733.3411) in the positive HR-FAB-MS. The 1H-NMR spectrum (Table 1) displayed a formyl signal at δ(H) 10.33 (s, H-19), an olefin CH signal at δ(H) 6.10 (s, H-22), O-bearing CH2 signals at δ(H) 5.25 (d, J = 18.4 Hz, H-21a) and 4.99 (d, J = 18.4 Hz, H-21b), O-bearing CH signal at δ(H) 4.64 (brs, H-3), and a tertiary CH3 at δ(H) 0.98 (s, H-18) indicating that 6 has a cardenolide skeleton. Also, two hemiacetal signals at δ(H) 5.07 (d, J = 7.6 Hz, H-1') and 4.69 (brd, J = 9.2 Hz, H-1'') were observed, and their large J values confirmed that the anemor hydroxyls were in β form. Two hexoses were determined to be β-digonopyransolyl-(1→4)-β-digonopyranose through comparisons between 13C-NMR data and those reported in previously published literature [15]. Acid hydrolysis of 6 and comparison of the specific rotation values of two isolated sugars [6a: [α]D20 = +55.5 (c = 0.12, H2O), 6b: [α]D20 = +49.3 (c = 0.12, H2O)] led to the identification of two sugars, d-digonopyranose ([α]D20 = +59.6) and d-glucopyranose ([α]D20 = +52.5) [16,17]. The locations of functional groups were determined by gCOSY and gHMBC experiments (Figure 2). Thus, compound 6 was identified as trophanthidin 3-O-β-D-digonopyransolyl-(1→4)-β-D-glucopyranoside, a new cardenolide named adoniside B.
Table 1. $^1$H- and $^{13}$C-NMR Data (400 and 100 MHz, resp.) of compounds 1 and 6.

| Position | 1 (CD$_3$OD) | 6 (C$_2$D$_3$N) |
|----------|--------------|-----------------|
|          | $\delta$(H) | $\delta$(C)     | $\delta$(H) | $\delta$(C) |
| 1        | 2.05 (m)     | 23.6            | 2.52 (m)    | 18.6        |
|          | 1.13 (m)     | 1.83 (m)        |             |             |
| 2        | 2.04 (m)     | 25.1            | 2.06 (m)    | 25.5        |
|          | 1.42 (m)     | 1.64 (m)        |             |             |
| 3        | 4.19 (br.s)  | 74.1            | 4.64 (br.s) | 74.5        |
| 4        | 1.94 (m)     | 35.2            | 2.27 (m)    | 37.0        |
|          | 1.62 (m)     | 1.81 (m)        |             |             |
| 5        |              | 73.5            |             | 73.8        |
| 6        | 1.93 (m)     | 35.9            | 2.17 (m)    | 36.0        |
|          | 1.57 (m)     | 1.76 (m)        |             |             |
| 7        | 1.51 (m)     | 21.4            | 1.52 (m)    | 24.8        |
|          | 1.43 (m)     | 1.32 (m)        |             |             |
| 8        | 1.96 (m)     | 41.4            | 2.26 (m)    | 41.9        |
| 9        | 1.41 (m)     | 39.0            | 1.64 (m)    | 39.5        |
| 10       |              | 54.3            |             | 55.2        |
| 11       | 2.26 (br.dd, $J = 14.8, 3.6$) | 18.1 | 2.43 (m) | 22.6 |
|          | 1.65 (m)     | 1.40 (m)        |             |             |
| 12       | 1.55 (m)     | 39.1            | 1.39 (m)    | 39.6        |
|          | 1.23 (m)     | 1.28 (m)        |             |             |
| 13       |              | 49.8            |             | 49.8        |
| 14       |              | 83.9            |             | 84.4        |
| 15       | 2.59 (dd, $J = 15.6, 9.6$) | 40.0 | 2.02 (m) | 32.2 |
|          | 1.74 (m)     | 1.79 (m)        |             |             |
| 16       | 5.41(dddd, $J = 9.6, 5.8, 2.8$) | 73.7 | 2.07 (m) | 27.2 |
| 17       | 3.15 (d, 8.4) | 55.6            | 2.76 (m) | 51.1 |
| 18       | 0.92 (s)     | 15.8            | 0.98 (s)    | 16.0        |
| 19       | 9.92 (s)     | 208.1           | 10.33 (s)   | 208.4       |
| 20       |              | 167.3           |             | 175.6       |
| 21       | 4.94 (dd, $J = 18.4, 1.6$) | 75.5 | 5.25 (d, $J = 18.4$) | 73.7 |
|          | 4.83 (dd, $J = 18.4, 1.6$) | 73.9         | 4.99 (d, $J = 18.4$) | 73.7 |
| 22       | 5.95 (dd, $J = 1.6, 1.6$) | 121.5         | 6.10 (s) | 117.8 |
| 23       |              | 173.9           |             | 174.4       |
| AcO      | 1.93 (s)     | 170.4           |             | 21.0        |
|          | Dgn (a)      | Dgn (a)         | D-Dig       |             |
| 1'       | 4.50 (dd, $J = 9.6, 2.4$) | 98.9 | 5.07 (brd, $J = 9.6$) | 99.5 |
| 2'       | 2.05 (m)     | 31.5            | 2.15 (m)    | 32.6        |
|          | 1.66 (m)     |                 |             |             |
| 3'       | 3.31 (dddd, $J = 12.8, 5.2, 2.0$) | 77.6 | 3.34 (brddd, $J = 12.0, 3.6$) | 79.7 |
| 4'       | 3.67 (br.s)  | 66.9            | 4.10 (br.s) | 73.9        |
| 5'       | 3.43 (brq, $J = 6.8$) | 70.7 | 3.48 (brq, $J = 6.4$) | 71.1 |
| 6'       | 1.33 (d, $J = 6.8$) | 16.7 | 1.49 (d, $J = 6.4$) | 17.8 |
| MeO      | 3.36 (s)     | 55.8            | 3.28 (s)    | 56.1        |
|          |              |                 | Glc (b)     |             |
| 1''      |              |                 |             | 104.9       |
| 2''      | 3.91 (dd, $J = 8.8, 7.6$) | 79.9 |
| 3''      | 4.16 (dd, $J = 8.8, 8.8$) | 78.5 |
| 4''      | 4.10 (dd, $J = 8.8, 8.8$) | 71.9 |
| 5''      | 3.89 (m)     | 78.3            |             |             |
| 6''      | 4.51 (dd, $J = 11.2, 1.6$) | 63.1 | 4.30 (dd, $J = 11.2, 6.0$) |             |

(a) $\beta$-d-diginopyranose; (b) $\beta$-d-glucopyranose $\delta$ in ppm, $J$ in Hz. Atom numbering as indicated in Figure 1.
All of the isolated cardenolides from *A. multiflora* were evaluated for cytotoxicity against six human cancer cell lines (HCT-116, HepG2, HeLa, SK-OV-3, SK-BR-3, and SK-MEL-5). As shown in Table 2, compounds 1, 2, 5, and 6 showed significant inhibition activity against HCT-116, SK-OV-3, and SK-MEL-5 cell lines with IC$_{50}$ values ranging from 0.06 ± 0.02 to 7.44 ± 1.98 µM. Compound 3 showed cytotoxic effects against the HeLa cell line with an IC$_{50}$ value of 8.85 ± 0.39 µM. Compound 4 showed cytotoxicity against the SK-MEL-5 cell line with an IC$_{50}$ value of 1.99 ± 0.28 µM.

Table 2. Cytotoxic activity of compounds 1–6 against human cancer cell lines (IC$_{50}$ [µM] $(a)$).

| Compound | Cell lines (IC$_{50}$) µM |
|----------|--------------------------|
|          | HCT-116 | HepG2 | HeLa | SK-OV-3 | SK-MEL-5 | SK-BR-3 |
| 1        | 4.10 ± 0.38 | 14.65 ± 0.47 | 38.54 ± 1.08 | 2.34 ± 0.10 | 3.40 ± 0.67 | 38.35 ± 1.49 |
| 2        | 0.41 ± 0.13 | 17.99 ± 0.61 | 9.38 ± 0.15 | 0.06 ± 0.02 | 0.28 ± 0.06 | 2.58 ± 0.23 |
| 3        | 34.99 ± 1.39 | 30.12 ± 1.60 | 8.85 ± 0.39 | 25.38 ± 0.51 | 34.17 ± 1.78 | 80.38 ± 1.13 |
| 4        | 24.32 ± 1.26 | 26.61 ± 0.70 | 23.27 ± 1.73 | 41.02 ± 0.13 | 1.99 ± 0.28 | 23.94 ± 1.47 |
| 5        | 1.64 ± 0.13 | 2.87 ± 0.77 | 25.38 ± 0.15 | 0.76 ± 0.15 | 0.73 ± 0.14 | 5.10 ± 0.87 |
| 6        | 7.44 ± 1.98 | 13.71 ± 0.75 | 44.71 ± 0.89 | 4.63 ± 0.47 | 4.98 ± 0.56 | 21.30 ± 1.50 |
| Doxorubicin | 9.20 ±0.90 | 27.30 ± 0.50 | 3.20 ± 0.30 | 0.58 ± 0.08 | 4.80 ± 0.23 | 0.71 ± 0.05 |

$(a)$ All data were represented as mean ± SD of triplicate experiments.

3. Experimental Section

3.1. General

Column chromatography (CC): SiO$_2$ (Kieselgel 60, Merck, Darmstadt, Germany) and ODS (LiChroprep RP-18, Merck) resins. TLC: Kieselgel 60 F$_{254}$ and RP-18 F$_{2545}$ (Merck) plates; visualization with UV lamp Spectroline Model ENF-240 C/F (Spectronics Corporation, Westbury, NY, USA) and spraying 10% H$_2$SO$_4$ soln. in MeOH and heating. Optical rotations: JASCO P-1010 digital polarimeter (Jasco, Tokyo, Japan). IR spectra: Perkin Elmer Spectrum One FT-IR spectrometer (Perkin Elmer, Beaconsfield, UK). FAB-MS: JEOL JMSAX-700 mass spectrometer (Jeol, Tokyo, Japan). NMR spectra: Varian Unity Inova AS-400 FT-NMR spectrometer (Varian, Palo Alto, CA, USA).
3.2. Plant Materials

*A. multiflora* Nishikawa & Koki Ito was supplied from the BMI Corporation (Uiwang, Korea) in January 2014, and was identified by professor Dae-Keun Kim, College of Pharmacy, Woosuk University, Jeonju, Korea. A voucher specimen (KHU2014-0117) has been reserved at the Laboratory of Natural Products Chemistry, Kyung Hee University, Yongin, Korea.

3.3. Extraction and Isolation

The whole plants of *A. multiflora* (1.5 kg) were extracted with 70% aqueous EtOH (30 L) at room temperature for 24 h. The concentrated EtOH extracts (106 g) were suspended in H$_2$O (3 L) and then successively extracted with CH$_2$Cl$_2$ (AAC-1; 2.6 g), AcOEt (AAC-2; 0.7 g), BuOH (AAC-3; 12 g), and H$_2$O (AAW; 89.2 g). The AAC (2.6 g) was subjected to CC [SiO$_2$ (ϕ 4 × 11 cm); CH$_2$Cl$_2$/MeOH 18:1, 15:1, 7:1, 1.6 L of each] yielding 16 fractions, AAC-1–AAC-16. Fr. AAC-3 (200 mg, elution volume/total volume (Ve/Vt) 0.03–0.06) was subjected to CC [ODS (ϕ 3 × 7 cm); MeOH/H$_2$O 3:1, 2.4 L], yielding 14 fractions, AAC-3-1–AAC-3-14. Fr. AAC-3-1 (52 mg, Ve/Vt 0.00–0.09) was subjected to CC [SiO$_2$ (ϕ 1.5 × 15 cm); Hexane/AcOEt 1:12, 0.5 L], yielding six fractions, AAC-3-1-1–AAC-3-1-6 along with a purified compound 1 [AAC-3-1-2; 12 mg; Ve/Vt 0.46–0.52; TLC (ODS F$_{254}$; MeOH/H$_2$O 5:2): $R_f$ 0.60]. Fr. AAC-4 (130 mg, Ve/Vt 0.06–0.08) was subjected to CC [ODS (ϕ 3 × 7 cm); MeOH/H$_2$O 4:5, 1.3 L], yielding 14 fractions, AAC-4-1–AAC-4-12 along with a purified compound 5 [AAC-4-9; 40 mg; Ve/Vt 0.63–0.81; TLC (ODS F$_{254}$; MeOH/H$_2$O 3:2): $R_f$ 0.45]. Fr. AAC-7 (200 mg, Ve/Vt 0.17–0.22) was subjected to CC [ODS (ϕ 3 × 5 cm); MeOH/H$_2$O 3:1, 1.6 L], yielding nine fractions, AAC-7-1–AAC-7-9 along with a purified compound 2 [AAC-7-2; 12 mg; Ve/Vt 0.05–0.07; TLC (ODS F$_{254}$; MeOH/H$_2$O 4:1): $R_f$ 0.45]. Fr. AAC-14 (121 mg, Ve/Vt 0.62–0.66) was subjected to CC [ODS (ϕ 3 × 6 cm); MeOH/H$_2$O 1:2, 2.7 L], yielding 11 fractions, AAC-14-1–AAC-14-11 along with a purified compound 3 [AAC-14-2; 8 mg; Ve/Vt 0.04–0.19; TLC (ODS F$_{254}$; MeOH/H$_2$O 2:1): $R_f$ 0.60], and compound 4 [AAC-14-6; 12 mg; Ve/Vt 0.53–0.69; TLC (ODS F$_{254}$; MeOH/H$_2$O 2:1): $R_f$ 0.50]. The AAB (12 g) was subjected to CC [SiO$_2$ (ϕ 7.5 × 16 cm); CH$_2$Cl$_2$/MeOH/H$_2$O 13:3:1, 9:3:1, 7:3:1, 65:35:10, 7 L of each] yielding 15 fractions, AAB-1–AAB-15. Fr. AAC-5 (300 mg, Ve/Vt 0.06–0.10) was subjected to CC [ODS (ϕ 2.5 × 5 cm); MeOH/H$_2$O 2:3, 1 L], yielding nine fractions, AAB-5-1–AAB-5-9 along with a purified compound 6 [AAB-5-7; 28 mg; Ve/Vt 0.38–0.71; TLC (ODS F$_{254}$; MeOH/H$_2$O 6:5): $R_f$ 0.30]..

3.4. Spectroscopic Data

*Adonioside A* (1). White powder. $[\alpha]_{D}^{20} = +23.9$ (c = 0.5, MeOH). IR (CaF$_2$): 3384, 2923, 1737, 1639, 1167, 1077 cm$^{-1}$. $^1$H- and $^{13}$C-NMR: Table 1. negative HR-FAB-MS: 605.2971 ([M − H]$^-$, C$_{32}$H$_{45}$O$_{11}$; calc. 605.2962).

*Adonioside B* (6). White powder. $[\alpha]_{D}^{20} = -94.4$ (c = 0.7, pyridine). IR (CaF$_2$): 3387, 2933, 1742, 1647, 1178, 1097 cm$^{-1}$. $^1$H- and $^{13}$C-NMR: Table 1. positive HR-FAB-MS: 733.3511 ([M + Na]$^+$, C$_{36}$H$_{54}$O$_{14}$Na; calc. 733.3414).

3.5. Acid Hydrolysis of 1 and 6

Compound 1 (10 mg) and compound 6 (20 mg) were refluxed in 2 N HCl (0.3 mL) at 80 °C for 5 h, followed by neutralization with Ag$_2$CO$_3$ in excess and filtered through filter paper. The filtrate of 1 was subjected to CC [SiO$_2$ (ϕ 1 × 10 cm); CHCl$_3$/MeOH 12:1] to give fractions of sugar (1a) and aglycone, and that of 6 was subjected to CC [SiO$_2$ (ϕ 1 × 10 cm); CHCl$_3$/MeOH 12:1, 1:1] to give fractions of sugars 6a, and 6b and aglycone. The monosaccharides 1a, 6a, and 6b in each sugar fraction were identified to be diginose, diginose, and glucose, respectively, by TLC comparison with authentic sugars. The $R_f$ values of diginose was 0.37 with CHCl$_3$/MeOH 9:1 and 0.47 with CH$_2$Cl$_2$/EtOH 9:1, and that of glucose was 0.30 with CHCl$_3$/MeOH/ H$_2$O 7:3:0.5 [18,19].
3.6. Determination of Absolute Configuration of 1a, 6a, and 6b

The sugar fractions, 1a (1 mg), 6a (1.2 mg), and 6b (1.2 mg), were measured for optical rotation values and compared with those reported in literature. Diginose, 1a and 6a, were determined to be D-form [1a: $\alpha_{20}^D = +56.8$ (c = 0.11, H$_2$O), 6a: $\alpha_{20}^D = +55.5$ (c = 0.12, H$_2$O); D-diginose: $\alpha_{20}^D = +59.6$]. Glucose 6b was determined to be D-form [6b: $\alpha_{20}^D = +49.3$ (c = 0.12, H$_2$O); D-glucose: $\alpha_{20}^D = +52.5$] [16,17].

3.7. Cell Culture

Human hepatoma (HepG2), human cervix adenocarcinoma (HeLa), human ovarian adenocarcinoma (SK-OV-3), human breast adenocarcinoma (SK-BR-3), human colon carcinoma (HCT-116), human melanoma (SK-MEL-5) cells were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). HepG2 and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin in a humidified incubator with 5% CO$_2$ at 37°C. SK-OV-3, SK-BR-3, HCT-116, and SK-MEL-5 cells were maintained in RPMI1640 medium containing 10% (v/v) heat-inactivated FBS and 1% (v/v) penicillin-streptomycin in a humidified incubator with 5% CO$_2$ at 37°C. All cell culture media and reagents were purchased from Thermo Scientific Hyclone (Logan, UT, USA).

3.8. Cytotoxicity Assay

The cytotoxicity of cardenolides from A. multiflora was measured by a MTT colorimetric assay. Compounds were dissolved with dimethylsulfoxide (DMSO). The cells were seeded onto 96-well microplates at a density of 1 x 10$^4$ cells per well in 100 µL of medium each. After incubation at 37 °C in a humidified incubator for 24 h, the cells were treated with various concentrations (1, 0.1, 0.5, 1, 5, 10, 50, 100 µM) of each compound in serum-free medium for 24 h. After incubation, 50 µL of MTT (5 mg/mL in PBS) was added to each well of the plate. The cells were incubated at 37 °C for 2 h. After removal from the medium, the cells were treated with 100 µL DMSO for 5 min and optical density measured using a microplate reader (BIO-TEK Inc., Winooski, VT, USA) at 550 nm. Cell viability was calculated as a percentage of viable cells in the compound-treated group vs. the control group by the following equation: Cell viability (%) = [OD (Compound) − OD (Blank)] / [OD (Control)−OD (Blank)] × 100.

3.9. Statistical Analysis

All experiments were performed with triplicate samples and repeated at least three times. The data are presented as means ± SD and statistical comparisons between groups were performed using 1-way ANOVA followed by Student’s t-test.

4. Conclusions

Two new and four known cardiac glycosides were isolated from the whole plants of Adonis multiflora Nishikawa & Koki Ito using open column chromatography and were identified based on spectroscopic data analysis, including NMR and FAB-MS. For the determination of absolute configuration, acid hydrolysis was performed. As a result, compound 1 and 6 were determined to be a 16-β-acetoxystrophanthidin 3-O-β-D-digonopyranoside, named adonioside A (1) and strophanthidin 3-O-β-D-digonopyranosyl(−1→4)-β-D-glucopyranoside, named adonioside B (6). In addition, the two new compounds 1 and 6 together with the two known compounds 2 and 5 showed significant cytotoxicity against six human cancer cell lines, HCT-116, HepG2, HeLa, SK-OV-3, and SK-MEL-5, and SK-BR-3, but we couldn’t establish a consistent structure-activity relationship. Consequently, these four compounds 1, 2, 5 and 6 merit further in vivo study and on normal cell lines for bioactive selectivity. These findings suggest that A. multiflora may have potential be a useful therapeutic natural source for cancer prevention.
**Supplementary Materials:** $^1$H-NMR and $^{13}$C-NMR spectra of 1 and 6 and HMBC spectrum of 6 are available as supporting data. Supplementary materials can be accessed at: http://www.mdpi.com/1420-3049/20/11/19722/s1.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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**Sample Availability:** Samples of the compounds 1–6 are available from the authors.

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