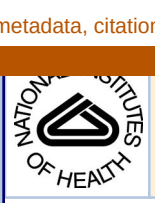

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Cytotoxic cardiac glycosides and coumarins from *Antiaris toxicaria*

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Abstract

Eight new cardiac glycosides/aglycones (antiaritoxiosides A–G, **1**–**7**, and antiarotoxinin B, **8**), two new coumarins (anticarins A–B, **41**–**42**), and two new flavanones (antiarones L–K, **43**–**44**) were isolated from trunk bark of *Antiaris toxicaria* together with 53 known compounds. The new structures were established by extensive analysis of spectroscopic data. Compound **1** (10-carboxy and 3α-hydroxy) and compounds **3**–**6** (10-hydroxy) contain unique substituents that are rarely found in cardiac glycosides. The cytotoxic effects of isolated compounds against ten human cancer cell lines, KB, KB-VIN, A549, MCF-7, U-87-MG, PC-3, 1A9, CAKI-1, HCT-9 and S-KMEL-2, were tested using the sulforhodamine B assay. Five compounds (**12**, **16**, **20**, **22**, and **31**) showed significant cytotoxicity against all ten cancer cell lines, with notable potency at the ng/mL level against some cell lines, which merits further development as clinical trial candidates.

Keywords

Antiaris toxicaria; Cardiac glycosides; Coumarins; Cytotoxicity

1. Introduction

Cardiac glycosides are naturally occurring compounds with a steroidal framework and are used for the treatment of congestive heart failure and as anti-arrhythmic agents. They also have been used as arrow poisons, abortifacients, emetics, diuretics, and heart tonics.[1]

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Mechanistically, their anti-arrhythmic activity results from inhibition of Na^+/K^+-ATPase, and leads to an increased intracellular calcium concentration. Recent research indicated that binding of cardiac glycosides to Na^+/K^+-ATPase can activate multiple downstream signal transduction pathways that regulate many important physiological and pathological states.[2] Less well known are the emerging role of this compounds category in the prevention and/or treatment of proliferative diseases, such as cancer. Because cardenolide-like compounds are often quite toxic, however, the potential use of such compounds for the treatment of cancer was abandoned 40 years ago. Even so, studies have revealed that some cardiac glycosides can selectively inhibit human tumor cell growth, but not normal cellular proliferation through complex cell signal transduction mechanisms.[1] Specifically, cardiac glycosides can inhibit growth of cancer cells, induce apoptosis via activation of caspase-3, induce cytochrome C release from mitochondria, and generate reactive oxygen species. In addition, clinical trials of cardiac glycosides and extracts containing them have been initiated to assess the anticancer potential.[2] Therefore, the use of certain cardiac glycosides may represent a worthwhile approach for control of cancer, despite their narrow therapeutic index.

In a previous investigation of bioactive compounds with potential therapeutic value, we reported cardiotonic principles from *Antiaris toxicaria* (Pers.) Lesch.[3] As a continuation of our anticancer research program based on natural products, an ethanolic extract of *A. toxicaria* (Moraceae) was found to show significant cytotoxicity against KB cells. *A. toxicaria*, also called ‘upas tree’, is widely distributed in tropical rain forests of Southeast Asia. Phytochemical studies have shown that this plant contains cardiac glycosides,[3,4] prenylaurones,[5] chalcones,[6] flavanones,[6] and dihydrochalcones.[7] Prior investigations also indicated that this species exhibited a broad spectrum of biological activities, such as anti-tumor,[8–12] anti-arrhythmic,[3] and 5-lipoxygenase inhibition effects.[12] Therefore, an intensive investigation of this cytotoxic extract was carried out and led to the isolation of 12 new compounds, together with 53 known compounds. Herein, we describe the isolation, structural elucidation, and antitumor activity of these isolates against ten human cancer cell lines. Preliminary structure–activity relationship (SAR) correlations are also described. In addition, based on the structures of 10-hydroxy-19-nor-cardenolides and 19-nor-cardenolides identified from this plant, a biogenetic pathway of cardiac glycoside has been proposed.

2. Results and discussion

Twelve new compounds, including eight cardiac glycosides/ aglycones (**1**–**8**), two coumarins (**41**–**42**) and two flavanones (**43**–**44**) (Fig. 1), together with 53 known compounds, were isolated from an EtOH extract of fresh trunk bark of *A toxicaria* through column chromatography on silica gel, Diaion HP-20, Sephadex H-20 and semipreparative HPLC. The subfractions were examined by H_2SO_4 solution spray on TLC for cardiac glycosides, which show as green spots, and subjected to a series of column chromatographic steps in order to obtain cardenolides/aglycones. The structures of new compounds were elucidated on the basis of spectroscopic methods, including 2D NMR techniques.

The HRFAB mass spectrum of **1** showed a molecular ion-related $[M+H]^+$ peak at *m/z* 567.2808, corresponding to the molecular formula $C_{29}H_{42}O_{11}$. The NMR signals were due

to one methyl, eleven methylene, eight methine and six quaternary carbons as determined by DEPT 135 spectroscopy (Table 1). Compound **1** showed a UV absorption maximum at 216 nm and IR absorption at 1734 cm $^{-1}$ (γ-lactone carbonyl), which were indicative of a butenolactone system.[13] The ^{1}H NMR spectrum of **1** (Table 2) showed characteristic signals for a butenolactone ring at δ_H 4.98 and 5.27 (each 1H, d, J = 18.1 Hz, H-21a, and b) and 6.08 (s, H-22), as well as a methyl singlet at δ_H 0.98 (s, H-18), suggesting **1** to be a cardenolide with a C-18 methyl group. A strong carbonyl absorption in the IR spectrum at 1734 cm^{-1} and a carbon signal at δ_C 176.7 in the ^{13}C NMR spectrum suggested that a carboxylic acid was present at C-19. Comparison of the ^{1}H and ^{13}C NMR spectra of **1** with those of antiaroside R (**9**),[4] which was isolated from the *n*-BuOH soluble fraction, showed that the two structures were very similar, except for the differences in glycosidic signals and orientation at C-3. An anomeric proton signal at δ_H 6.08 (s) in the ^{1}H NMR spectrum and signals at δ_C 99.8, 72.7, 72.8, 74.3, 69.9, and 18.6 in the ^{13}C NMR spectrum indicated the presence of α-rhamnose. The α-orientation of C-3 was deduced from the coupling constant values of H-3 (dddd, J = 11.0, 11.0, 5.4, 5.4 Hz). This assignment was supported by downfield shifts of H-2a, H-3, and H-4a from δ_H 1.43 (m), 4.37 (br s, $W_{1/2}$ = 15.7 Hz), and 1.67 (d, J =12.7 Hz) in **9** to δ_H 2.19 (m), 4.81 (dddd, J = 11.0, 11.0, 5.4, 5.4 Hz), and 2.19 (m) in **1**. HMBC correlation of H-1′ with C-3 [δ_C 72.9/δ_H 6.08 (s)] inferred that the rhamnose unit was linked to C-3. Hence, the structure shown was established for **1** (antiaritoxioside A).

A molecular formula of $C_{28}H_{42}O_{10}$ was deduced for compound **2**, 12 mass units less than that of periplorhamnoside (**11**).[14] The ^{1}H and ^{13}C NMR data of the two compounds were similar except for the C-19 and C-10 signals. The carbon signal at δ_C 17.2 (C-19) in **11** was absent in **2.** Furthermore, the carbon signal at δ_C 41.2 (C-10) in **11** was present at δ_C 74.4 (C-10) in **2**. Thus, both the NMR and MS data were indicative of a hydroxy rather than methyl group at C-10 in **2**. The β-orientation of the C-3 monosaccharide unit was deduced from the $W_{1/2}$ constant of H-3 (br s, $W_{1/2}$ = 6.6 Hz). Therefore, the structure of **2** was determined as demethyl-10-hydroxy-periplorhamnoside and **2** was named antiaritoxioside B.

Compound **3** showed a pseudo molecular ion peak at *m/z* 539.2855 in its HRFABMS and had the same molecular formula, $C_{28}H_{42}O_{10}$, as toxicarioside M (**10**),[15] which was also isolated from the *n*-BuOH extract of this plant. The UV, IR and NMR data of **3** strongly resembled those of **10**, consistent with a general structure containing a central cardenolide moiety dioxygenated at C-3, and C-5 and a hydroxy group in the 10-position. The sole significant differences were in certain glycosidic signals (Tables 1 and 2). An anomeric proton signal at δ_H 5.40 (d, J = 5.8 Hz) in the ^{1}H NMR spectrum and signals at δ_C 99.3, 72.2, 73.1, 73.8, 70.8, and 18.8 in the ^{13}C NMR spectrum indicated the presence of β-allomethylose. An NOE effect from H-1′ to H-3 suggested that the allomethylose unit was attached to C-3. These data agreed with the replacement of the antiarosyl unit in **10** by an allomethylosyl unit in **3**. Thus, the structure of **3** was established for antiaritoxioside C.

A molecular formula of $C_{28}H_{43}O_{11}$ was deduced for compound **4**, 12 mass units less than that of β-antiarin (**34**),[16] isolated from the $CHCl_3$ soluble fraction. The ^{1}H and ^{13}C NMR data of the two compounds were similar except for the absence of a C-19 aldehyde group

and a downfield shift of C-10 from δ_C 55.0 in **34** to δ_C 74.7 in **4**. These data suggested that the aldehyde group in **34** was replaced by a hydroxy group in **4**. This conclusion was supported by $^3J_{CH}$ HMBC correlations from H-1b and H-11b to C-10. The β-orientations of the C-3 and -12 OH groups were deduced from the coupling constants of H-3 (δ_H 4.27, br. s, $W_{1/2}$ = 7.9 Hz) and H-12 (δ_H 3.74, br t, J = 7.8 Hz). Thus, **4** was determined as 10-hydroxy-19-*nor*-β-antiarin and named antiaritoxioside D.

Compound **5** had the same molecular formula, $C_{28}H_{43}O_{11}$, as **4**. The UV, IR and NMR data also strongly resembled those of **4**, consistent with a structure containing a 19-*nor*-cardenolide moiety tetraoxygenated at C-3, C-5, C-10 and C-12. The sole significant differences occurred in glycosidic signals of the molecules (Tables 1 and 2). The sugar proton signals of **5** indicated the presence of a β-antiarosyl moiety. These data were consistent with the replacement of the rhamnosyl unit in **5** by an antiarosyl unit in **5**. The β-orientations of C-3 and C-12 were deduced by the $W_{1/2}$ of H-3 (br s, 7.7 Hz) and H-12 coupling constant (br t, J = 9.7 Hz). Thus, the structure of **5** was assigned as shown, and **5** was named antiaritoxioside E.

The HRFAB mass spectrum of **6** showed a molecular ion-related $[M+Na]^+$ peak at *m/z* 529.2774, corresponding to the molecular formula $C_{28}H_{42}O_8$. The ^1H and ^{13}C NMR data (Tables 1 and 2) indicated that **6** was a 19-nor-cardenolide monosaccharide with a hexose sugar unit. Analyses of COSY, HMQC and HMBC spectra indicated that the sugar was α-rhamnose. Comparison of the ^1H and ^{13}C NMR spectra of **6** with those of malayoside (**29**),[3,16] showed that the two structures were very similar, except for that **6** did not contain the C-19 aldehyde group found in **29** based on the presence of a methine (−CH) proton at δ_H 1.43 (m), upfield shift of C-10 from δ_C 51.2 to δ_C 34.0, and absence of CHO signals. Compound **6** was thus assigned to be 19-nor-malayoside and named antiaritoxioside F.

Compound **7** ($C_{29}H_{45}O_{10}$) was 16 mass units larger than **11**.[14] The $^1H/^{13}C$ NMR spectra of **7** and **11** were very similar, except for the sugar moiety signals and one oxygenated carbon in **7**. The spectra of **11** showed a proton signal at δ_H 2.81 (dd, J = 8.9, 5.1 Hz) and a carbon signal at δ_C 51.4 (C-17), while in **7**, the former signal was absent and the latter signal was replaced by an oxygenated carbon signal at δ_C 87.8 (C-17). Thus, an OH group was attached at C-17 in **7**, and this postulate was supported by a downfield shift of C-16 from δ_C 27.35 in **11** to δ_C 34.3 in **7**, together with HMBC correlations of H-18 to C-17. An anomeric proton signal at δ_H 5.39 (d, J = 8.1 Hz) and carbon signals at δ_C 99.8, 69.6, 73.8, 73.7, 70.0, and 17.3 indicated the presence of an antiarosyl moiety. An NOE effect between H-3 and H-1′ placed the antiarosyl unit on C-3, and a $W_{1/2}$ coupling constant of 9.8 Hz for H-3 indicated α-orientation. Therefore, the structure **7** was established for antiaritoxioside G.

Compound **8** ($C_{21}H_{36}O_5$) had 21 signals in the ^{13}C NMR spectrum corresponding to two methyl, ten methylene, five methine, and four quaternary carbon atoms. The ^1H and ^{13}C NMR spectra of **8** displayed signals characteristic of the steroid skeleton of a cardenolide. However, the absence of typical signals for the olefinic group and carbonyl carbon of the butenolactone ring, suggested that the five-membered lactone in **8** was absent. This hypothesis was confirmed by the presence of a methylene group at δ_C 65.8/ δ_H 3.89 (1H, dd, J = 10.5, 6.1 Hz) and 4.08 (1H, dd, J = 10.5, 7.2 Hz, H-21) and one methine at δ_C 71.1/δ_H

4.36 (1H, dd, *J* = 7.2, 6.1 Hz, H-20), as well as the absence of any significant UV absorption. In addition to three OH groups on the steroid skeleton (C-3, C-5 and C-14), two OH groups were assigned at C-20 and C-21, based on carbon signals at δ_C 71.1 and 65.8, respectively. Orientation of the C-3 OH was also determined by the $W_{1/2}$ of H-3 (br s, $W_{1/2}$ = 11.3 Hz). The above analysis established the structure of **8** as shown. This compound was reported as an intermediate in the synthesis of periplogenin by Peoghenghi et al.[17] It has been isolated from a natural source for the first time and named antiarotoxinin B.

Compound **41** was obtained as colorless syrup, and its molecular formula was determined to be $C_{15}H_{18}O_6$ on the basis of HREIMS ($[M]^+$, *m/z* 294.1100). The IR spectrum showed absorption bands at 3400 and 1711 cm^{-1} for a hydroxyl group and α, β-unsaturated lactone, respectively. UV absorptions at 316 and 258 nm,[18] and two AB type system protons at δ_H 7.94 and 6.32 (each 1H, d, *J* = 9.6 Hz, H-4 & -3) suggested that **41** was a coumarin derivative.[19] The ^{1}H NMR spectrum of **41** also showed an aromatic proton at δ_H 7.06 (1H, s), three hydroxy protons at δ_H 9.60, 4.39 and 4.23 (each 1H, D_2O exchangeable), one methoxy group at δ_H 3.79 (3H, s) and one 2,3-dihydroxyisopentyl group at δ_H 1.12 and 1.10 (each 3H, s), 3.36 (1H, m), 2.95 (1H, d, *J* = 13.2 Hz) and 2.39 (1H, dd, *J* = 13.2, 10.4 Hz). The ^{13}C NMR spectrum of **41** revealed the presence of 15 carbons resonances including two methyl, one methoxy, one methylene, four methine and seven quaternary carbons. Comparison of the ^{1}H and ^{13}C NMR spectra of **41** with those of skimminan, which was isolated from *Skimmia anquetelia* by Sharma et al.,[20] showed that the two structures were very similar, except for the absence of signals for a sugar and one additional methoxy group (δ_H 3.79, δ_C 60.5) in the former. The HMBC spectrum showed correlations of the methoxy group at δ_H 3.79 with C-7 (δ_C 149.9) and of H-1′ (δ_H 2.95, and 2.39) with C-5 (δ_C 119.7), C-6 (δ_C 131.0) and C-7 (δ_C 149.9). Therefore, the methoxy group was attached at C-7 and the 2, 3-dihydroxyisopentyl moiety was located at C-6. This assignment was further confirmed by NOE effects between H-4 (δ_H 7.94) and H-5 (δ_H 7.06), as well as H-5 and H-1′ for **41**. From the above data, new compound **41** was elucidated as 8-hydroxy-7-methoxy-6-[2′, 3′-dihydroxyisopentyl]coumarin, and has been designated as anticarin A.

Compound **42**, $[\alpha]_D^{25}$ +38.2° (*c* 0.25, MeOH), was isolated as colorless needles. The HRFABMS exhibited a pseudo-molecular ion at *m/z* 425.1367 for $[M+H]^+$, consistent with the molecular formula $C_{20}H_{24}O_{10}$. UV absorptions at 326, 302 (sh), 259, 247 and 220 (sh) nm,[18] and IR band at 1709 (OC=O) cm^{-1}, as well as two AB type protons at δ_H 7.96 (1H, d, *J* = 9.2 Hz, H-4) and 6.26 (1H, d, *J* = 9.2 Hz, H-3) suggested that **42** was a coumarin derivative.[19] In the ^{1}H NMR spectrum, the presence of two additional AB type protons at δ_H 7.54 and 6.76 (each 1H, d, *J* = 8.8 Hz), two vicinal methine protons at δ_H 4.84 (1H, d, *J* = 2.4 Hz) and 3.94 (1H, dd, *J* = 4.4, 2.4 Hz), and two methyl groups at δ_H 1.27 (6H, s), indicated that **42** was a khellactone glycoside.[21] The signal at δ_H 4.84 was assigned as H-4′ based on its correlations to C-7 (δ_C 156.6), C-9 (δ_C 155.0), C-8 (δ_C 108.6), and C-1″ (δ_C 103.8) in the HMBC spectrum, whereas the signal at δ_H 3.94 was assigned as H-3′ based on its correlation to C-3′ in the HMQC spectrum, as well as long range correlations of two methyl groups (δ_H 1.27) to C-3′ (δ_C 70.6). An anomeric proton signal at δ_H 4.71 (1H, d, *J* = 8.0 Hz, H-1″) in the ^{1}H NMR spectrum and signals at δ_C 103.8, 74.0, 76.8, 70.3, 77.3 and 61.5 in the ^{13}C NMR spectrum suggested the presence of β-glucose. The coupling constant

of the anomeric doublet (δ_H 4.71, 8.0 Hz) suggested a β-glucosidic linkage for the khellactone. The FAB-MS spectrum showed an ion fragment at *m/z* 263[M^+-162], also indicating the presence of a hexose moiety. The β-glucose unit was placed at C-4′ on the basis of HMBC correlation between H-1″ of the glucose unit and C-4′ of the aglycone moiety, together with NOE correlation between H-1″ and H-4′. Accordingly, the structure of **42** was established as 4′-β-glucosyl-khellactone, and **42** has been named anticarin B.

Compound **43** was obtained as colorless obtained as colorless needles. The molecular formula formula was established as $C_{21}H_{22}O_6$ from *m/z* 370.1412 $[M]^+$ in HREIMS. The IR spectrum of **43** showed absorption bands at 3456 and 3175 cm^{-1} for one or more hydroxy group(s) and 1638 cm^{-1} for a conjugated carbonyl functionality. The presence of a flavanone skeleton was evident from UV [226 (sh), 288, and 328 (sh) nm],[18] and ^1H [δ_H 2.66 (1H, *dd*, *J* =17.2, 2.8 Hz, H-3α), 3.16 (1H, *dd*, *J* = 17.2, 13.2 Hz, H-3β) and 5.62 (1H, *dd*, *J* = 13.2, 2.8 Hz, H-2)] and ^{13}C [δ_C 79.9 (C-2), 43.1 (C-3) and 197.4 (C-4)] NMR spectra. The ^1H NMR spectrum further showed three hydroxy groups [δ_H 12. 17, 9.69 and 7.55 (each 1H, s)], two singlet aromatic protons [δ_H 5.96 (2H, s)], AB system aromatic protons [δ_H 6.90 (1H, d, *J* =8.4 Hz) and 7.06 (1H, d, *J* = 8.4 Hz)], a methoxy moiety [δ_H 3.86 (3H, s)], and an isoprenyl group [δ_H 1.67 (3H, s), 1.63 (3H, s), 3.52 (2H, d, *J* = 6.4 Hz) and 5.13 (1H, t, *J* = 6.4 Hz)]. The locations of these substituents were determined by HMQC and HMBC experiments. Based on ^3J-correlations between the proton at δ_H 5.96 with C-5 (δ_C 165.1), C-7(δ_C 167.2), and C-10 (δ_C 103.0) in the HMBC experiment and with C-6 in a HMQC spectrum, the singlet aromatic protons were assigned to H-6 and H-8, respectively, of ring-A HMBC correlations of the proton at δ_H 6.90 to C-1′ (δ_C 130.5) and C-3′ (δ_C 144.8) and at δ_H 7.06 to C-2 (δ_C 79.9) and C-2′ (δ_C 126.9) suggested that the AB system aromatic protons belonged H-5′ and H-6′, respectively. Combined with HMBC correlations of H-1″ (δ_H 3.52) to C-1′ (δ_C 130.5), C-2′ (δ_C 126.9), C-3′ (δ_C 144.8), and C-2″ (δ_C 124.0), and methoxy protons (δ_H 3.86) to C-4′ (δ_C 148.0) implied that the isoprenyl group was attached to C-2′, while the methoxy group was located at C-4′. These assignments were further confirmed by an NOE effect between the methoxy group and H-5′ (δ_H 6.90). Finally, a hydroxy group was attached to C-3′. The *S*-configuration of C-2 was determined by a positive Cotton effect observed at 312 nm and a negative one at 286 nm in the circular dichroism (CD) spectrum of **43**.[22] Based on these observations, **43** was established as (2*S*)-3′,5,8-trihydroxy-4′-methoxy-6,7-methylenedioxy-2′-prenylflavanone, and named antiarone L.

The molecular formula of **44** was determined as $C_{25}H_{26}O_6$ from a molecular ion peak at *m/z* 423.1733 $[M+H]^+$ in its HRESIMS. The UV (324, 286, and 231 nm),[18] ^1H, and ^{13}C NMR spectra indicated that **44** was also a flavanone. The ^1H and ^{13}C NMR spectra of **44** closely resembled those of **43** except that signals for a 2,2-dimethylpyran ring unit were present at δ_H 6.40 (1H, d, *J* = 9.8 Hz, H-4‴), 5.73 (1H, d, *J* = 9.8 Hz, H-3‴), 1.20 and 1.18 (6H, s, 2‴-CH_3) and δ_C 122.7 (C-4‴), 131.3 (C-3‴), 77.5 (C-2‴), 29.1 & 32.4 (2‴-CH_3) in **44**, and the methoxy group found **43** was absent. Correlations of H-1″ [δ_H 3.48 (2H, d, *J* = 6.8 Hz)] to C-1′ (δ_C 130.1), C-2′ (δ_C 124.0) and C-3′ (δ_C 143.8) in the HMBC spectrum indicated an isoprenyl group was attached to C-2′, whereas long range correlations of H-4‴ (δ_H 6.40) to C-4′ (δ_C 140.5), C-5′ (δ_C 133.1) and C-6′ (δ_C 120.0) and of H-3‴ (δ_H 5.73) to C-5′

suggested that the 2,2-dimethylpyran ring was fused to C-5′ and 4′. The absolute stereochemistry of **44** was deduced to be 2S because of a positive Cotton effect at 314 nm and a negative one at 290 nm in the circular dichroism (CD) spectrum of **44**.[22] Thus, the structure of compound **44** was assigned as (2*S*)-2‴,2‴-dimethylpyrano-3′,5,8-trihydroxy-2′-prenylflavanone, and named antiarone K.

Antiaroside R (**9**),[4] toxicarioside M (**10**),[15] periplorhamnoside (**11**),[14] antiaroside A (**12**),[3] peripalloside (**13**),[14] cheiranthoside (**14**),[23] strophanthidol (**15**),[24] convallatoxal (**16**),[16] cannogenol (**17**),[25] antiaroside B (**18**),[3] strophanthidin (**19**),[14] convallatoxin (**20**),[16] strophathojavoside (**21**),[26] desglucocheirotoxin (**22**),[24] strophalloside (**23**),[14] convalloside (**24**),[27] glucostrophalloside (**25**),[28] cheirotoxin (**26**),[29] antiaroside C (**27**),[3] antiaroside D (**28**),[3] malayoside (**29**),[3,16] antiarigenin (**30**),[14] α-antiarin (**31**),[16] antialloside (**32**),[26] toxicarioside B (**33**),[30] β-antiarin (**34**),[16] antiaroside E (**35**),[3] antiaroside F (**36**),[3] antiaroside G (**37**),[3] antiaroside H (**38**),[3] antiaroside I (**39**),[3] antiarotoxinin A (**40**),[3] (+)-marmesin (**45**),[31], decursinol (**46**),[21] (*R*)-peucedanol (**47**),[32] oxypeucedanin hydrate (**48**),[33] (2*S*)-pinocembrin (**49**),[34] antiarones F (**50**),[6] I (**51**),[6] vanillic acid (**52**),[35] *p*-hydroxybenzoic acid (**53**),[36], vanillin (**54**),[35] (3*S*,4*R*)-hydroxymellein (**55**),[37] 2, 4,6-trihydroxy-5-methoxyactophenone (**56**),[38] 6-acetoxy-2,4-dihydroxyacetophenone (**57**),[39] syringic aicd (**58**),[40] koaburaside (**59**),[41] vanilloyl β-D-glucose (**60**),[42] uridine (**61**),[43] xanthine (**62**),[44] adenosine (**63**),[36] 3-hydroxy-5-methoxybenzalacetophenone (**64**),[45] and β-sitosterol (**65**)[35] were also isolated and identified by comparison of their physical and spectroscopic properties with those reported in the literature.

Cardenolides **11–40** and coumarins **41**, **42**, and **45** were assayed for anti-proliferative effects against 10 human cancer cell lines, KB, KB-VIN, A549, MCF-7, U-87-MG, PC-3, 1A9, CAKI-1, HCT-9, and S-KMEL-2.[46] The cytotoxic data are shown in Table 3. Among the tested isolates, compounds **11**, **12**, **16**, **17**, **19–25**, **29**, **31–35**, and **42** exhibited potent effects against all cancer cell lines. Among them, compounds **12**, **16**, **20**, **22**, and **31** showed ng/mL potency against one or more cancer cell lines. Compound **12** exhibited an ED_{50} of 0.0097 μg/mL against KB cancer cell lines. Compound 16 demonstrated ED_{50} values of 0.0040, 0.0076, and 0.0061 μg/mL against A549, 1A9 and CAKI-1 cells, respectively. Compound **20** showed ED_{50} values of 0.0027, 0.0065, 0.0031, 0.0014 and 0.0013 μg/mL against A549, MCF-7, CAKI-1, HCT-9 and s-KMEL-2 cells, respectively. Compound **22** exhibited an ED_{50} of 0.0092 μg/mL against A549 cells. Finally, compound **31** showed an ED_{50} of 0.0044 μg/mL against PC-3 cells. Common structural features in all five compounds included a 5β-OH, 13β-Me, 14β-OH, and 17β-α, β-unsaturated-γ-butyrolactone. Compounds **20**, **22**, and **31** contained a C-18 β-CHO, while **12** and **16** contained β-CH_3 and −CH_2OH, respectively. Compound **31** contained a C-12 β-OH, while compounds **12**, **16**, **20**, and **22** were unsubstituted. All five compounds contained a 3β-sugar moiety, 3β-*O*-β-D-antiarose in **12**, **22**, and **31**, 3β-*O*-α-L-rhamnose in **16** and **20**.

The following specific structure-activity correlations were noted in these studies. Changing the orientation of the glycoside from 3β in potent cardenolide **20** to 3α in **27** significantly decreased the cytotoxicity, for example, from 0.0027 to 0.48 μg/mL against A549, from 0.0065 to 1.4 against MCF-7, from 0.0031 to 0.31 μg/mL against CAKI-1, from 0.0014 to

1.5 μg/mL against HCT-9, and from 0.0013 to 0.26 μg/mL against s-KMEL-2. Compound **28** with a 3α-diglycoside was also one of the least potent cardenolides. Activity decreased dramatically when the 3β-*O*-α-L-rhamnose of **16** (ED_{50} values 0.0.0040–0.036 μg/mL) was changed to 3β-OH in **15** (ED_{50} values 0.14–6.5 μg/mL). Replacement of the C-10 CH_3 of **12** with COOH in **38** together with addition of a 12β-OH decreased cytotoxicity from 0.0097 to 5.9 μg/mL against KB cells. Similarly, changing the C-10 CH_2OH of **16** to H in **39** decreased cytotoxicity from 0.0040 to 0.36 μg/mL against A549 cells. Compounds showing the lowest potency against the cancer cell lines included **14**, **15**, **27**, and **28** with ED_{50} values ranging from 0.12 to 4.2 μg/mL, as well as **30** and **36–40** with ED_{50} values of 0.36–17.5 μg/mL. Notably, a C-10 COOH, COO-glucose, or H was found in **36–39** rather than CHO, CH_3, or CH_2OH as found in the more potent compounds. While the cytotoxic effects of new compounds **1–6** were not examined in this report,[46] the cytotoxic effects of structurally similar cardiac glycosides have been reported.[4,10] Our observations were generally consistent with the prior findings (for example, compound **9** with COO-glucose at C-10 and compounds with α-oriented substituents at C-3 exhibited lower cytotoxicity).[4] In summary, from our investigation, the β-orientation of C-3, C-3 monosaccharide, and C-10 substituents (CHO, CH_3, or CH_2OH) are crucial factors in the cytotoxicity effects of cardiac glycosides. Prior reports also indicated that compounds with C-10 OH may exhibit significant cytotoxicity.[10]

Among the three tested coumarins, compound **41** and the structurally related linear dihydrofuranocoumarin **45** showed no cytotoxic activity, whereas the angular dihydropyranocoumarin **42** exhibited broad and significant cytotoxicity against KB-VIN, A549, MCF-7, PC-3, 1A9, CAKI-1 and S-KMEL-2 cell lines with ED_{50} values ranging from 1.2 to 4.1 μg/mL.

It is a definite possibility that the structurally similar cardiac glycoside metabolites were derived biogenetically from a general catabolic process. A possible biogenetic transformation pathway is presented in Scheme 1 for the first time on the basis of evidence from natural sources. The proposed biosynthesis could explain the catabolic progression of structurally interesting cardiac glycosides.

3. Conclusions

In summary, this paper described the isolation and structure elucidation of 12 new compounds, including eight cardiac glycosides/aglycones, antiaritoxiosides A-G (**1–7**) and antiarotoxinin B (**8**), two coumarins, anticarin A–B, and two flavanones, antiarones L–K. The cytotoxic activity of cardiac glycosides and coumarins against ten cancer cell lines also reported. Compounds **12**, **16**, **20**, **22**, and **31** showed significant cytotoxicity against all 10 cancer cell lines, with notable potency at the ng/mL level against some cell lines. SAR correlations were also identified. While many cardiac glycosides are reported in the literature, compounds with 10-carboxy (e.g., **1**, **9**) and 3α-hydroxy (e.g., **1**) substituents are rare. Furthermore, the C-10 substituents, including COOH, CHO, CH_2OH, CH_3, OH and H, of cardiac glycosides can help to explain the catabolic progression of structurally interesting cardiac glycosides.

4. Experimental

4.1. General procedures

Optical rotations were measured with a JASCO DIP-370 polarimeter. IR spectra were determined as KBr discs on a Shimazu FTIR-8501 spectrophotometer. UV spectra were acquired on a Hitachi UV-3210 spectrophotometer. The ^{1}H and ^{13}C NMR spectra were recorded on Bruker Avance 300 (^{1}H at 300 MHz) and AMX 400 (^{1}H at 400 MHz) spectrometers. The ^{1}H and ^{13}C chemical shifts (ppm) were referenced to the solvent peaks for pyridine-d_5 at δ_H 8.71 and δ_C 149.9, DMSO-d_6 at δ_H 2.49 and δ_C 39.5, acetone-d_6 at δ_H 2.05 and δ_C 29.9, 206.7, and methanol-d_4 at δ_H 3.31 and δ_C 49.0. ESIMS, FABMS and HRMS were taken with a VG 70–250 S spectrometer by direct inlet system. Merck silica gel 60 (Merck 70–230, 230–400 mesh) was used for column chromatography. Glass sheets of silica gel 60 F_{254} (Merck 0.2 mm thick) were used for TLC Melting points were measured on a Yanagimoto MP-S3 micromelting point apparatus and are uncorrected.

4.2. Plant material

Trunk bark of *A toxicaria* was collected from Yunnan, China, and authenticated by C.S. Kuoh (Department of Life Sciences, National Cheng Kung University, Tainan, Taiwan). A voucher specimen (NCKUWu 92012) has been deposited in the Herbarium of National Cheng Kung University, Tainan, Taiwan, R.O.C.

4.3. Extraction and isolation

Trunk bark of *A toxicaria* (6.0 kg) was cut into small pieces and extracted with 95% EtOH (20 L × 3). Evaporation of the solvent under reduced pressure provided 239.0 g of crude extract, which was partitioned between $CHCl_3$−H_2O, and *n*-BuOH−H_2O, successively, to yield $CHCl_3$ (AT-C, 65.1 g), *n*-BuOH (AT-B, 100.2 g), and H_2O (AT-W, 73.7 g) fractions. The $CHCl_3$ fraction was subjected to silica gel CC using increasing polarity mixtures of *n*-hexanes-acetone as eluant to give 14 fractions (AT-C-1 ∼ 14). AT-C-2 was recrystallized to obtain **65** (42.5 mg). AT-C-3 was chromatographed on silica gel using *n*-hexane–diisopropyl ether (3:1) to obtain **56** (1.2 mg), **57** (1.7 mg), and **64** (11.5 mg). AT-C-4 was chromatographed on silica gel using *n*-hexane–diisopropyl ether (3:1) to obtain **43** (12.4 mg), **49** (3.1 mg), **50** (10.0 mg), **51** (175.4 mg), **55** (1.2 mg), and **64** (2.4 mg). AT-C-5 was chromatographed on silica gel eluted with *n*-hexane–diisopropyl ether (1:1) to give **43** (18.0 mg), **44** (1.0 mg), **49** (3.7 mg), **50** (17.1 mg), and **55** (2.4 mg). AT-C-6 was chromatographed on silica gel using diisopropyl ether−MeOH (40:1) to obtain **7** (8.7 mg), **19** (103.2 mg), **46** (3.1 mg), **52** (8.7 mg), **54** (12.3 mg), and **65** (12.5 mg). AT-C-7 was chromatographed on silica gel and eluted with *n*-hexane–diisopropyl ether (1:1) to give **15** (6.6 mg) and **21** (7.0 mg). AT-C-8 was chromatographed on silica gel using EtOAc−MeOH (20:1) to obtain **6** (2.1 mg), **8** (19.6 mg), **17** (3.2 mg), **29** (8.8 mg), **35** (37.6 mg), and **39** (7.5 mg). AT-C-9 and 10 were chromatographed on silica gel using EtOAc−MeOH (20:1) to obtain **29** (21.4 mg), and **20** (117.2 mg), respectively.

The *n*-BuOH fraction was subjected to Diaion HP-20 CC eluting with a H_2O−MeOH gradient system to give 12 fractions (AT-B-1 ∼ 12). AT-B-4 and 5 were chromatographed on silica gel using $CHCl_3$−MeOH (9:1) to obtain **4** (1.3 mg), **5** (1.5 mg), **30** (58.9 mg), **31**

(11.0 mg), **32** (4.3 mg), **34** (7.3 mg), **37** (34.7 mg), **38** (147.4 mg), **52** (10.0 mg), **53** (10.2 mg), and **54** (5.5 mg), successively. AT-B-6 was chromatographed on silica gel using $CHCl_3$−MeOH (9:1) to obtain **31** (118.4 mg). AT-B-7 and 8 were chromatographed on silica gel using $CHCl_3$−MeOH−H_2O (9:1:005) to obtain **1** (3.0 mg), **2** (3.8 mg), **10** (0.5 mg), **14** (9.1 mg), **16** (6.1 mg), **18** (12.3 mg), **20** (431.2 mg), **22** (4.3 mg), **23** (5.9 mg), **24** (91.2 mg), **25** (18.3 mg), **26** (0.5 mg), **27** (112.3 mg), **28** (12.2 mg), **36** (5.9 mg), **41** (25.3 mg), **42** (5.8 mg), **47** (6.7 mg), and **58** (2.7 mg), successively. AT-B-9 and 10 were chromatographed on silica gel using $CHCl_3$−MeOH−H_2O (9:1:0.05) to obtain **3** (0.3 mg), **11** (10.9 mg), **12** (9.8 mg), **13** (1.9 mg), **20** (12.5 mg), **22** (29.9 mg), **23** (17.8 mg), **33** (13.5 mg), **40** (0.7 mg), **45** (1.2 mg), **48** (0.9 mg), and **53** (2.8 mg), successfully.

The H_2O fraction was subjected to Diaion HP-20 CC eluting with a H_2O−MeOH gradient system to give 10 fractions (AT-W-1 ∼ 10). AT-W-2 and 3 were chromatographed on silica gel using EtOAC−MeOH−H_2O (4:1:0.5) to obtain **61** (2.3 mg), and **62** (10.7 mg). AT-W-4 was chromatographed on silica gel using EtOAC−MeOH−H_2O (4:1:0.5) to obtain **59** (22.4 mg), **60** (29.0 mg), and **63** (1.2 mg). AT-W-5 was chromatographed on silica gel using $CHCl_3$−MeOH−H_2O (4:1:0.005) to obtain **59** (12.6 mg). AT-W-6 and 7 was chromatographed on silica gel using EtOAC−MeOH−H_2O (4:1:0.5) to obtain **58** (17.3 mg). AT-W-8 and 9 were chromatographed on Sephadex LH-20 using a H_2O−MeOH gradient system to obtain **9** (0.7 mg), **20** (43.2 mg), **24** (26.8 mg), **28** (26.4 mg), **31** (20.2 mg) and **32** (1.3 mg), successfully.

4.4. Antiaritoxioside A (1)

Colorless syrup; $[\alpha]_D^{25}$ +189.13° (*c* 0.01 in MeOH); UV λ_{max} (MeOH)/nm (log ε) 216 (4.21); IR ν_{max}/cm^{-1} (KBr) 3400, 2930, 1734, 1651, 1506, 1456, 1394, 1229, 1026; ^{1}H NMR data (pyridine-d_5) see Table 2; ^{13}C NMR data (pyridine-d_5) see Table 1; FABMS *m/z* 567 (13%) $[M+H]^+$; HRFABMS *m/z* 567.2808 ($C_{29}H_{43}O_{11}$ $[M+H]^+$ requires 567.2805).

4.5. Antiaritoxioside B (2)

Colorless syrup; $[\alpha]_D^{25}$ −9.52° (*c* 0.04 in MeOH); UV λ_{max}(MeOH)/nm (log ε) 215 (4.31); IR ν_{max}/cm^{-1} (KBr) 3440, 2937, 1744, 1647, 1634, 1454, 1032; ^{1}H NMR data (pyridine-d_5) see Table 2; ^{13}C NMR data (pyridine-d_5) see Table 1; FABMS *m/z* 539 (8%) $[M+H]^+$; HRFABMS *m/z* 539.2855 ($C_{28}H_{43}O_{10}$ $[M+H]^+$ requires 539.285).

4.6. Antiaritoxioside C (3)

Colorless syrup; $[\alpha]_D^{25}$ −242.2° (*c* 0.003 in MeOH); UV λ_{max} (MeOH)/nm (log ε) 213 (4.14); IR ν_{max}/cm^{-1} (KBr) 3410, 2928, 1734, 1647, 1636, 1541, 1456, 1076, 1036; ^{1}H NMR data (pyridine-d_5) see Table 2; ^{13}C NMR data (pyridine-d_5) see Table 1; FABMS *m/z* 539 (6%) $[M+H]^+$; HRFABMS *m/z* 539.2855 ($C_{28}H_{43}O_{10}$ $[M+H]^+$ requires 539.2856).

4.7. Antiaritoxioside D (4)

Colorless syrup; $[\alpha]_D^{25}$ −45.88° (*c* 0.01 in MeOH); UV λ_{max} (MeOH)/nm (log ε) 214 (4.17); IR ν_{max}/cm^{-1} (KBr) 3410, 2932, 1742, 1649, 1454, 1377, 1029; ^{1}H NMR data (pyridine-d_5)

see Table 2; ^{13}C NMR data (pyridine-d_5) see Table 1; FABMS *m/z* 555 (3%) $[M+H]^+$; HRFABMS *m/z* 555.2805 ($C_{28}H_{43}O_{11}$ $[M+H]^+$ requires 555.2805).

4.8. Antiaritoxioside E (5)

Colorless syrup; $[\alpha]_D^{25}$ −69.72° (*c* 0.02 in MeOH); UV λ_{max} (MeOH)/nm (log ε) 271 (3.63), 218 (4.36); IR ν_{max}/cm^{-1} (KBr) 3410, 2945, 1732, 1640, 1630, 1452, 1385, 1171, 1109, 1078,1028, 997; ^{1}H NMR data (pyridine-d_5) see Table 2; ^{13}C NMR data (pyridine-d_5) see Table 1; FABMS *m/z* 555 (8%) $[M+H]^+$; HRFABMS *m/z* 555.2802 ($C_{28}H_{43}O_{11}$ $[M+H]^+$ requires 555.2805).

4.9. Antiaritoxioside F (6)

Colorless syrup; $[\alpha]_D^{25}$ −15.28° (*c* 0.02 in MeOH); UV λ_{max} (MeOH)/nm (log ε) 216 (4.32); IR ν_{max}/cm^{-1} (KBr) 3410, 2926, 1742, 1645, 14474, 1377, 1049; ^{1}H NMR data (pyridine-d_5) see Table 2; ^{13}C NMR data (pyridine-d_5) see Table 1; FABMS *m/z* 529 (7%) $[M+Na]^+$, 507 (5%) $[M+H]^+$; HRFABMS *m/z* 529.2774 ($C_{28}H_{42}$-NaO_8 $[M+Na]^+$ requires 529.2777).

4.10. Antiaritoxioside G (7)

Colorless powder ($CHCl_3$−MeOH); mp 227–228 °C; $[\alpha]_D^{25}$ −57.69° (*c* 0.01 in MeOH); UV λ_{max} (MeOH)/nm (log ε) 215 (4.62); IR ν_{max}/cm^{-1} (KBr) 3350, 2949, 1753, 1383, 1286, 1175, 1101, 1022, 991; ^{1}H NMR data (pyridine-d_5) see Table 2; ^{13}C NMR data (pyridine-d_5) see Table 1; FABMS *m/z* 553 (2%) $[M+H]^+$; HRFABMS *m/z* 553.3014 ($C_{29}H_{45}O_{10}$ $[M+H]^+$ requires 553.3012).

4.11. Antiarotoxinin B (8)

Colorless powder ($CHCl_3$−MeOH); mp >280 °C; $[\alpha]_D^{25}$ −31.34° (*c* 0.02 in MeOH); IR ν_{max}/cm^{-1} (KBr) 3261, 2936, 2882, 1470, 1448, 1383, 1088, 1047; ^{1}H NMR data (pyridine-d_5) see Table 2; ^{13}C NMR data (pyridine-d_5) see Table 1; EIMS *m/z* 368 (8%) $[M]^+$, 350 (28%), 319 (39%), 301 (41%), 296 (100%), 290 (15%), 283 (20%), 272 (14%), 147 (12%), 121 (15%), 107 (28%), 97 (39%); HREIMS *m/z* 368.2560 ($C_{21}H_{36}O_5$ $[M]^+$ requires 368.2563).

4.12. Anticarin A (41)

Colorless syrup; $[\alpha]_D^{25}$ +16.3° (*c* 0.058, MeOH); UV λ_{max} (MeOH)/nm (log ε) 316 (3.60), 258 (3.48); IR ν_{max}/cm^{-1} (KBr) 3400, 2926, 1711, 1607, 1572, 1450, 1279; ^{1}H NMR (400 MHz, DMSO-d_6) δ_H 9.60 (1H, br s, D_2O exchangeable, OH), 7.94 (1H, d, *J* = 9.6 Hz, H-4), 7.06 (1H, s, H-5), 6.32 (1H, d, *J* = 9.6 Hz, H-3), 4.39 (1H, d, *J* = 6.0 Hz, D_2O exchangeable, 2′-OH), 4.23 (1H, br s, D_2O exchangeable, 3′-OH), 3.79 (3H, s, OCH_3), 3.36 (1H, m, H-2′), 2.95 (1H, d, *J* = 13.2 Hz, H-1′), 2.39 (1H, dd, *J* = 13.2, 10.4 Hz, H-1′), 1.12 (3H, s, H-4′), 1.10 (3H, s, H-5′); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C 160.3 (C-2), 149.9 (C-7), 145.0 (C-4), 142.3 (C-9), 137.2 (C-8), 131.0 (C-6), 119.7 (C-5), 115.0 (C-10), 114.0 (C-3), 77.8 (C-2′), 71.9 (C-3′), 60.5 (OCH3), 31.4 (C-1′), 26.6 (C-5′), 24.8 (C-4′); EIMS *m/z* 294 (28%) $[M]^+$, 261 (7%), 236 (79%), 235 (37%), 221 (23%), 205 (100%), 193 (56%), 177 (20%),

163 (25%), 147 (22%), 59 (84%); HREIMS *m/z* 294.1100 ($C_{15}H_{18}O_6$ $[M]^+$ requires 294.1103).

4.13. Anticarin B (42)

Colorless needles (MeOH); mp. 265–267 °C; $[\alpha]_D^{25}$ +38.2° (*c* 0.2523, MeOH); UV λ_{max} (MeOH)/nm (log ε) 326 (4.18), 302 (3.92, sh), 259 (3.46), 247 (3.50), 220(4.13, sh), 204 (4.67); IR ν_{max}/cm^{-1} (KBr) 3300, 1709, 1605, 1592, 1541, 1306, 1126, 1076, 1036; ^1H NMR (400 MHz, DMSO-d_6) δ_H 7.96 (1H, d, *J* = 9.2 Hz, H-4), 7.54 (1H, d, *J* = 8.8 Hz, H-5), 6.76 (1H, d, *J* = 8.8 Hz, H-6), 6.26 (1H, d, *J* = 9.2 Hz, H-3), 5.29 (1H, d, *J* = 4.4 Hz, D_2O exchangeable, 3′-OH), 4.93 (2H, m, D_2O exchangeable, 3″, 4″-OH), 4.84 (1H, br. d, *J* = 2.4 Hz, H-4′), 4.71 (1H, d, *J* = 8.0 Hz, H-1″), 4.52 (1H, br s, D_2O exchangeable, 4′-OH), 4.42 (1H, t, *J* = 6.0 Hz, D_2O exchangeable, 6″-OH), 3.94 (1H, dd, *J* = 4.4, 2.4 Hz, H-3′), 3.76 (1H, br. dd, *J* = 12.0, 6.0 Hz, H-6″), 3.50 (1H, br s dd, *J* = 12.0, 6.0 Hz, H-6″), 3.25 (1H, m, H-5″), 3.22 (1H, t, *J* = 8.4 Hz, H-3″), 3.05 (1H, m, H-4″), 2.90 (1H, t, *J* = 8.4 Hz, H-2″), 1.27 (6H, s, 20-CH_3 × 2); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C 160.1 (C-2), 156.6 (C-7), 155.0 (C-9), 144.8 (C-4), 129.1 (C-5), 114.1 (C-6), 112.0 (C-3, 10), 108.6 (C-8), 103.8 (C-1″), 78.5 (C-2′), 77.3 (C-5″), 76.8 (C-3″), 74.0 (C-2″), 71.4 (C-4′), 70.6 (C-3′), 70.3 (C-4″), 61.5 (C-6″), 24.9 & 24.3 (2′-CH_3 × 2); FABMS *m/z* 425 (23%) $[M+H]^+$, 371 (8%), 307 (10%), 263 (75%), 245 (31%), 219 (74%), 154 (100%), 136 (86%); HRFABMS *m/z* 425.1367 ($C_{20}H_{25}O_{10}$ $[M+H]^+$ requires 425.1369).

4.14. Antiarone L (43)

Colorless needles (acetone); mp. 131–133°C; $[\alpha]_D^{25}$ −3.0° (*c* 0.53, MeOH); UV λ_{max} (MeOH)/nm (log ε) 328 (3.63, sh), 288 (4.31), 226 (4.44, sh), 206 (4.86); IR ν_{max}/cm^{-1} (KBr) 3456, 3175, 2964, 2924, 1638, 1605, 1497, 1460, 1375, 1277, 1163, 1082; CD (*c* 5.4 × 10^{-5}, MeOH) $[\theta]_{208}$ −9580, $[\theta]_{224}$ +38450, $[\theta]_{286}$ −22450, $[\theta]_{312}$ +52330; ^1H NMR (400 MHz, acetone-d_6) δ_H 12.17 (1H, br s, D_2O exchangeable, 5-OH), 9.69 (1H, br s, D_2O exchangeable, 8-OH), 7.55 (1H, br s, D_2O exchangeable, 3′-OH), 7.06 (1H, d, *J* =8.4 Hz, H-60), 6.90 (1H, d, *J* = 8.4 Hz, H-5′), 5.96 (2H, s, H-6&8), 5.62 (1H, dd, *J* = 13.2, 2.8 Hz, H-2), 5.13 (1H, t, *J* = 6.4 Hz, H-2″), 3.86 (3H, s, OCH_3), 3.52 (2H, d, *J* = 6.4Hz, H-1″), 3.16 (1H, dd, *J* = 17.2, 13.2 Hz, H-3α), 2.66 (1H, dd, *J* = 17.2, 2.8 Hz, H-3β), 1.67&1.63 (each 3H, s, H-4″&5″); ^{13}C NMR (acetone-d_6, 100 MHz) δ_C 197.4 (C-4), 167.2 (C-7), 165.1 (C-5), 164.4 (C-9), 148.0 (C-4′), 144.8 (C-3′), 131.4 (C-3″), 130.5 (C-1′), 126.9 (C-2′), 124.0 (C-2″), 118.2 (C-6′), 109.5 (C-5′), 103.0 (C-10), 96.7 (C-8), 95.7 (C-6), 79.9 (C-2), 56.2 (OCH_3), 43.1 (C-3), 25.7 & 17.9 (C-4″ & 5″), 25.0 (C-1″); EIMS *m/z* 370 (100%) $[M]^+$, 352 (10%), 219 (12%), 153 (26%), 125 (18%); HREIMS *m/z* 370.1412 ($C_{21}H_{22}O_6$ $[M]^+$ requires 370.1416).

4.15. Antiarone K (44)

Colorless powder (acetone); mp 171–173 °C; $[\alpha]_D^{25}$ −44.7° (*c* 0.001, MeOH); UV λ_{max} (MeOH)/nm (log ε) 324 (3.41, sh), 286 (4.06), 231 (4.35, sh), 213 (4.25); IR ν_{max}/cm^{-1} (KBr) 3600–3000, 2963, 2926, 2855, 1639, 1522, 1466, 1456, 1340, 1271, 1161, 1085; CD (*c* 5.0 × 10^{-5}, MeOH) $[\theta]_{210}$ −10580, $[\theta]_{229}$ +39460, $[\theta]_{290}$ −25380, $[\theta]_{314}$ +52190; ^1H

NMR (acetone-d_6, 400 MHz) δ_H 12.18 (1H, br s, D_2O exchangeable, 5-OH), 7.70 (1H, br s, D_2O exchangeable, 3′-OH), 6.56 (1H, s, H-6′), 6.40 (1H, d, J = 9.8 Hz, H-1‴), 5.95 (2H, s, H-6 & 8), 5.73 (1H, d, J = 9.8 Hz, H-2‴), 5.60 (1H, dd, J = 13.4, 2.6 Hz, H-2), 5.13 (1H, t, J = 6.8 Hz, H-2″), 3.48 (2H, d, J = 6.8 Hz, H-1″), 3.17 (1H, dd, J = 17.0, 13.4Hz, H-3α), 2.64 (1H, dd, J = 17.0, 2.6 Hz, H-3β), 1.63&1.67 (each 3H, s, H-4″&5″), 1.18 & 1.20 (each 3H, s, 2‴-CH_3 × 2); ^{13}C NMR (acetone-d_6, 100 MHz) δ_C 197.4 (C-4), 167.2 (C-7), 165.2 (C-5), 164.5 (C-9), 143.8 (C-3′), 140.5 (C-4′), 133.1 (C-5′), 131.3 (C-3″ & 3‴), 120.1 (C-1′), 127.1 (C-2′), 124.0 (C-2″), 122.7 (C-4‴), 120.0 (C-6′), 103.0 (C-10), 96.7 (C-8), 95.7 (C-6), 77.5 (C-2‴), 77.0 (C-2), 43.2 (C-3), 32.4 & 29.1 (C-2‴-CH_3 × 2), 25.7&17.9 (C-4″& 5″), 25.2 (C-1″); EIMS m/z 436 (100%) $[M]^+$; 418 (10%), 286 (25%), 255 (18%); 153 (26%); HREIMS m/z 436.1889 ($C_{26}H_{28}O_6$ $[M]^+$ requires 436.1886).

4.16. In vitro cytotoxicity assay

The sulforhodamine B assay was used according to the procedures developed and validated at NCI.[47] Doxorubicin was used as the positive control antitumor drug. The in vitro anticancer activities are expressed as ED_{50} values, which is the test compound concentration (μg/mL) that reduced the cell number by 50% after 72 h of continuous treatment. The values were interpolated from dose-response data. Each test was performed in triplicate with variation less than 5%. The ED_{50} values determined in each of independent tests varied less than 10%. Compound stock solutions were prepared in DMSO with the final solvent concentration ≤ 1% DMSO (v/v), a concentration without effect on cell replication. The cells were cultured at 37 °C in RPMI-1640 supplemented with 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 2% (w/v) sodium bicarbonate, 10% (v/v) fetal bovine serum, and 100 μg/mL kanamycin in a humidified atmosphere containing 5% CO_2.

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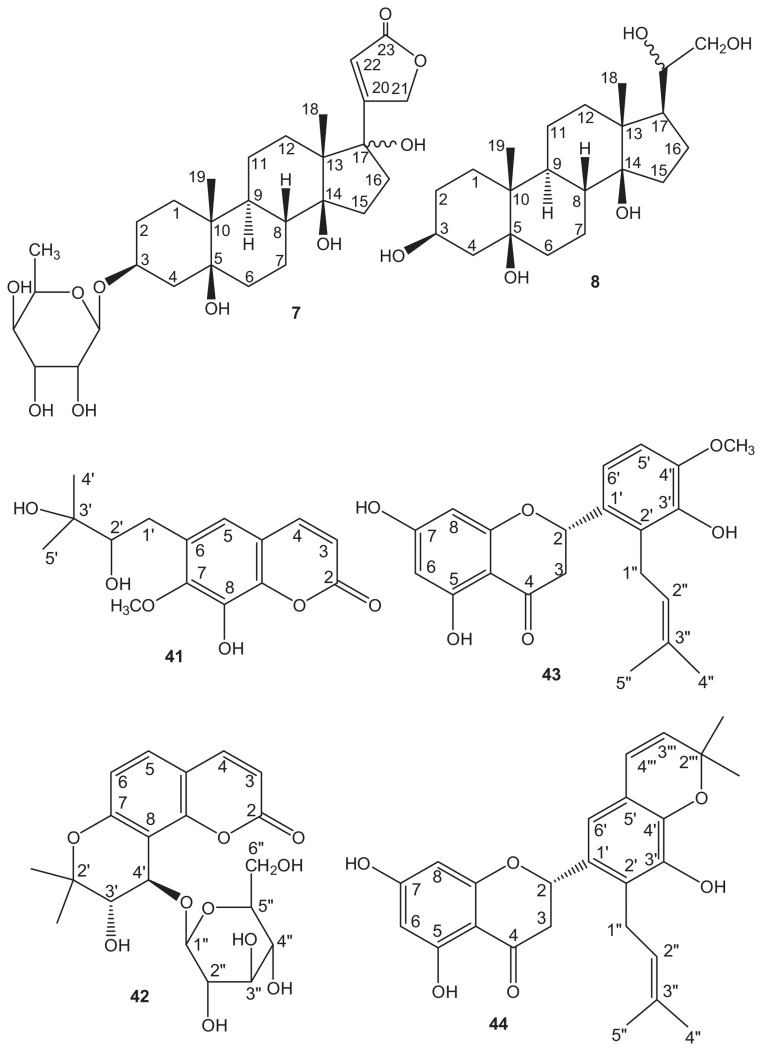
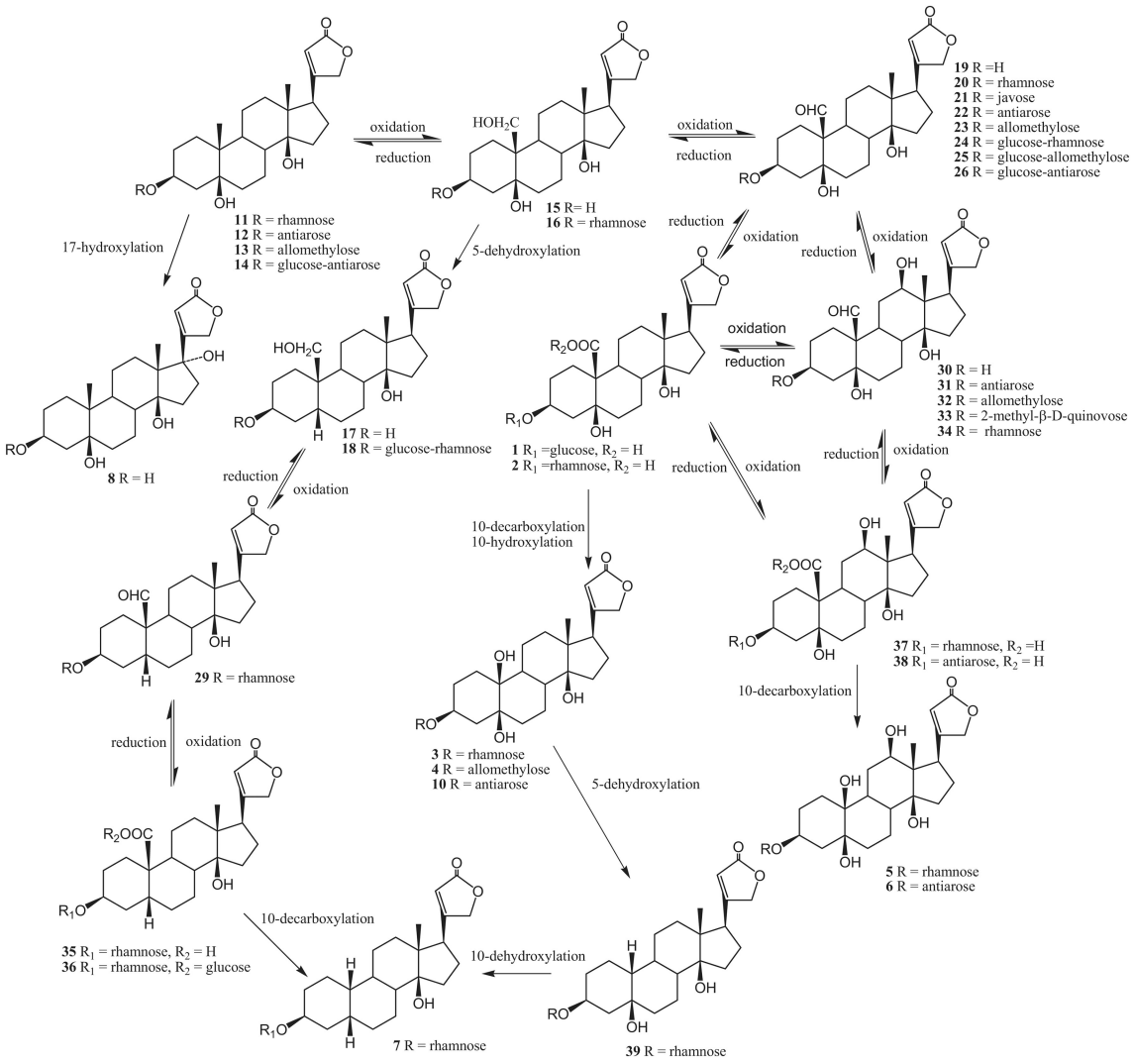


Figure 1.
Chemical structures for new compounds.



Scheme 1.
Biosynthetic pathway of cardiac glycosides.

Table 1

^{13}C NMR data for antiaritoxioside A-G (1–7) and antiarotoxinin B (8)

Carbon	1‡	2‡	3‡	4‡	5	6	7‡	8
1	36.3	26.9	25.3	26.9	26.7	22.0	23.6	25.8
2	22.4	28.7	27.4	28.6	27.9	26.4	26.8	28.6
3	72.9	73.9	73.8	73.8	73.2	72.3	74.1	67.8
4	32.1	35.6	34.8	35.6	35.1	30.0	36.1	36.1
5	72.9	74.7	73.1	74.2	74.3	30.4	73.4	74.5
6	43.3	35.8	35.1	35.9	36.5	33.2	40.8	37.8
7	28.4	24.3	24.5	24.8	24.7	26.0	21.3	24.4
8	42.3	40.7	41.3	40.4	40.4	48.2	41.2	39.0
9	41.0	40.2	39.4	37.5	37.7	42.0	37.3	40.0
10	55.7	74.4	74.4	74.7	74.4	34.0	39.2	41.3
11	23.5	21.6	22.8	30.8	30.7	22.1	26.1	22.0
12	39.9	40.0	40.4	74.7	74.7	39.7	31.4	40.3
13	50.2	50.1	50.3	56.7	56.9	50.1	51.9	47.8
14	84.8	84.6	85.0	85.0	85.1	83.8	86.7	83.8
15	33.4	33.0	33.0	33.4	33.4	32.2	33.7	33.1
16	27.2	27.5	27.2	27.9	29.2	22.1	34.3	19.0
17	51.5	51.3	51.3	46.4	46.2	51.4	87.8	51.5
18	16.3	16.3	16.3	10.3	10.3	16.4	13.1	15.5
19	176.7						17.0	17.4
20	176.1	176.3	176.4	176.7	176.8	176.0	174.0	71.1
21	73.8	73.8	73.8	74.0	74.1	73.6	73.8	65.8
22	117.6	117.7	117.6	117.5	117.4	117.6	116.6	
23	174.6	174.8	174.6	174.7	174.8	174.5	173.2	
1′	99.8	100.8	99.3	100.7	99.4	99.8	99.8	
2′	72.7	72.6	72.2	72.6	69.8	72.9	69.6	
3′	72.8	73.0	73.1	72.9	73.6	72.9	73.8	
4′	74.3	73.9	73.8	73.7	73.7	74.1	73.3	
5′	69.9	70.7	70.8	70.7	70.0	70.0	70.0	

Carbon	1‡	2‡	3‡	4‡	5	6	7‡	8
6′	18.6	18.6	18.8	18.6	17.0	18.6	17.3	

δ Value in pyridine-d_5 (100 MHz).

‡ δ Value in pyridine-d_5 (75 MHz).

Table 2

^{1}H NMR data for antiaritoxioside A-G (1–7) and antiarotoxinin B (8)

Proton	1	2‡	3	4	5	6	7	8
1a/b	1.76 (br d, 13.6)/3.29 (ddd, 13.6, 13.6, 5.7)	1.90 (m)/2.19 (m)	2.04 (m)/2.44 (m)	1.95 (m)/2.27 (br dd, 8.4, 2.3)	1.92 (m)/2.46 (br s dd, 14.5, 2.9)	1.23 (m)/2.07 (m)	1.32 (ddd, 13.5, 13.5, 3.8)/2.21 (m)	1.45 (t, 11.7)/2.21 (td, 12.5, 4.4)
2a/b	2.19 (m)/2.27 (m)	1.67 (m)/2.04 (m)	1.81 (m)/2.14 (m)	1.69 (br d, 14.8)/2.00 (m)	1.81 (br d, 13.1)/2.17 (m)	1.52 (m)	1.92 (m)/2.07 (m)	1.81 (m)
3	4.81 (dddd, 11.0, 11.0, 5.4, 5.4)	4.29 (br s, 6.6)†	4.40 (br s, 15.7)†	4.27 (br s, 7.9)†	4.48 (br s, 7.7)†	4.18 (br s, 9.9)†	4.52 (br s, 9.8)†	4.44 (br s, 11.3)
4a/b	2.19 (m)/2.45 (br d, 12.8)	1.90 (m)/2.11 (m)	1.81 (m)/2.04 (m)	1.97 (m)/2.15 (m)	1.92 (m)/2.09 (br d, 9.7)	1.23 (m)/1.48 (br t, 4.0)	1.56 (m)/1.97 (m)	1.78 (m)/2.32 (dd, 14.9, 2.5)
5						2.07 (m)		
6a/b	2.19 (m)/2.27 (m)	1.55 (m)/2.25 (m)	1.65 (m)/2.14 (m)	1.56 (dd, 11.2, 6.3)/ 1.97 (m)	1.63 (br d, 13.0)/2.28 (m)	1.78 (m)/2.02 (m)	1.49 (m)/2.07 (m)	1.62 (br d, 13.0)/ 2.03 (m)
7a/b	2.27 (m)/2.68 (br d, 11.8)	1.23 (m)/2.33 (br. d, 9.8)	1.65 (m)/2.15 (m)	1.32 (br d, 13.2)/ 2.29 (br s dd, 9.9, 3.3)	2.12 (m)/2.32 (m)	1.55 (m)/1.78 (m)	1.30 (m)/1.64 (br t, 13.5)	1.40 (m)/2.36 (td, 13.3, 2.0)
8	2.27 (m)	2.36 (d, 12.3)	2.15 (m)	2.48 (br t, 11.8)	2.48 (br dd, 12.8, 2.9)	1.29 (m)	1.99 (m)	1.76 (dt, 12.2, 11.8)
9	2.27 (m)	1.63 (m)	1.65 (m)	1.78 (ddd, 8.7, 8.7, 3.0)	1.81 (br d, 13.1)	1.24 (m)	2.39 (m)	2.03 (m)
10						1.43 (m)		
11a/b	1.88 (m)	1.55 (m)/1.90 (m)	1.38 (m)/2.26 (m)	2.15 (m)/2.24 (m)	2.12 (m)/2.25 (m)	1.66 (br d, 2.3)/1.84 (m)	1.84 (br t, 13.9)/2.25 (m)	1.40 (m)
12a/b	1.46 (m)	1.40 (m)	1.38 (m)	−/3.74 (br t, 7.8)	−/3.76 (br t, 9.7)	1.31 (m)	2.17 (m)/ 2.39 (m)	1.42 (t, 11.9)
15a/b	1.93 (m)/2.19 (m)	1.90 (m)/2.04 (m)	1.81 (m)/2.04 (m)	1.73 (m)/1.97 (m)	1.92 (m)	1.35 (m)	1.19 (m)/1.49 (m)	1.89 (m)/2.09 (dt, 12.6, 9.8)
16a/b	1.93 (m)/2.03 (dd, 20.0, 10.8)	2.04 (m)	1.81 (m)/2.04 (m)	2.15 (m)	2.12 (m)	1.94 (m)/2.07 (m)	1.97 (m)/2.21 (m)	1.92 (m)/2.26 (m)
17	2.76 (dd, 9.1, 3.2)	2.78 (br d, 7.7)	2.79 (br t,2.5)	3.76 (br t, 7.4)	3.76 (br t, 9.7)	2.76 (br d, 8.8)		2.21 (m)
18	0.98 (s)	1.05 (s)	1.15 (s)	1.28 (s)	1.29 (s)	0.99 (s)	1.26 (s)	1.31 (s)
19							1.17 (s)	1.19 (s)
20								4.36 (t, 6.5)
21a/b	4.98 (d, 18.1)/5.27 (d, 18.1)	5.13 (d, 18.2)/5.30 (d, 18.2)	5.01 (d, 18.0)/5.29 (d, 18.0)	5.09 (d, 17.9)/5.23 (d, 17.9)	5.03 (d, 18.1)/5.33 (d, 18.1)	5.02 (d, 17.8)/5.31 (br d, 17.8)	5.07 (dd, 18.2, 1.4)/ 5.21 (dd, 18.2, 1.4)	3.89 (dd, 10.5, 6.1)/ 4.08 (dd, 10.5, 7.2)
22	6.08 (s)	6.12 (s)	6.12 (s)	6.22 (s)	6.23 (s)	6.12 (s)	6.23 (d, 1.4)	
1′	5.44 (br s)	5.49 (br s)	5.40 (d, 5.8)	5.33 (br s)	5.43 (d, 8.1)	5.41 (br s)	5.39 d, 8.1)	

Proton	1	2‡	3	4	5	6	7	8
2′	4.27 (br s)	4.54 (br s)	3.96 (d, 4.7)	4.48 (br s)	4.50 (dd, 8.1, 3.1)	4.54 (m)	4.46 (br d, 8.1)	
3′	4.44 (dd, 8.9, 3.4)	4.44 (dd, 9.0, 3.2)	4.67 (m)	4.38 (dd, 8.6, 2.8)	4.77 (t, 3.1)	4.54 (m)	4.70 (m)	
4′	4.19 (dd, 8.9, 8.9)	4.31 (m)	3.66 (d, 6.2)	4.21 (br t, 8.6)	4.13 (d, 3.1)	4.29 (m)	4.11 (br s)	
5′	4.32 (dq, 8.9, 6.1)	4.22 (m)	4.34 (dq, 6.2, 4.6)	4.18 (dq, 8.6, 5.9)	4.61 (q, 6.6)	4.29 (m)	4.59 (q, 6.4)	
6′a/b	1.59 (d, 6.1)	1.63 (d, 6.0)	1.59 (d, 4.6)	1.61 (d, 5.9)	1.53 (d, 6.6)	1.67 (d, 4.7)	1.56 (d, 6.4)	

δ Value in pyridine-d_5 (400 MHz), coupling constants in Hz are given in parentheses.

† $W_{1/2}$ (Hz): width of 1/2 peak high.

‡ δ Value in pyridine-d_5 (300 MHz), coupling constants in Hz are given in parentheses.

Table 3

Cytotoxicity (ED_{50} μg/mL) of isolated compounds from *A. toxicaria*[a]

Compound	KB*	KB-VIN	A549	MCF-7	U-87-MG	PC-3	1A9	CAKI-1	HCT-9	S-KMEL-2
11	0.054	0.020	0.012	0.018	0.082	0.021	0.012	0.014	0.044	0.017
12	0.0097	0.068	0.044	0.063	0.15	0.078	0.020	0.018	0.090	0.073
14	1.5	1.7	0.12	0.58	1.9	0.60	0.53	0.51	0.94	0.16
15	2.7	3.0	0.14	0.69	2.9	0.81	0.66	0.67	6.4	0.57
16	0.025	0.014	0.0040	0.012	0.036	0.013	0.0076	0.0061	0.020	0.014
17	0.45	0.50	0.050	0.21	0.50	0.15	012	0.11	0.46	0.70
19	0.61	0.69	0.11	0.38	0.72	0.17	0.15	0.14	0.51	0.13
20	0.014	0.013	0.0027	0.0065	0.014	0.094	0.030	0.0031	0.0014	0.0013
21	0.066	0.051	0.016	0.042	0.078	0.052	0.013	0.014	0.067	0.064
22	0.044	0.018	0.0092	0.015	0.054	0.018	0.012	0.014	0.044	0.015
23	0.081	0.050	0.015	0.024	0.043	0.063	0.013	0.014	0.085	0.063
24	0.2	0.18	0.070	0.15	0.40	0.095	0.072	0.071	0.20	0.42
25	0.12	0.083	0.038	0.096	0.15	0.084	0.057	0.065	0.13	0.12
27	1.5	1.6	0.48	1.4	1.7	0.42	0.39	0.32	1.5	0.26
28	1.7	1.9	0.38	1.0	4.2	0.53	0.74	0.93	1.9	0.31
29	0.30	0.32	0.080	0.19	0.37	0.11	0.076	0.073	0.18	0.62
30	5.3	5.1	0.59	1.3	4.6	1.2	0.12	1.3	4.5	0.41
31	0.14	0.15	0.059	0.10	0.017	0.0044	0.058	0.072	0.15	0.13
32	0.065	0.075	0.046	0.12	0.15	0.085	0.062	0.068	0.11	0.12
33	0.13	0.14	0.058	0.092	0.13	0.091	0.063	0.070	0.11	0.12
34	0.067	0.061	0.013	0.018	0.071	0.050	0.014	0.015	0.066	0.065
35	0.49	0.57	0.070	0.42	0.73	0.16	0.13	0.13	0.35	0.12
36	1.8	2.2	0.54	1.3	4.5	0.89	0.97	1.2	3.4	0.32
37			5.3	17.5						
38	5.9	7.1	0.76	3.1	7.3	1.8	1.4	1.5	2.5	1.3
39	2.0	2.3	0.36	1.3	3.6	0.90	0.83	1.1	2.9	0.30
40			4.3	15.0						
41			10.8	>20		1.5	1.5	1.5	6.7	1.3

Compound	KB*	KB-VIN	A549	MCF-7	U-87-MG	PC-3	1A9	CAKI-1	HCT-9	S-KMEL-2
42	5.8	4.1	1.2	3.1	7.4					
45			>20	>20						

[a] Compounds **1**–**9** were not tested due to insufficient quantities remaining.

[*] Cell line, ED_{50} in μg/mL.