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Original Research Article

Chemical Constituents of Dichloromethane Extract from the Leaves of *Gardenia angkorensis*, their Cytotoxic and *α*-Glucosidase Inhibition Activities

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ABSTRACT

Gardenia is a genus of about 140 different plant species of the Rubiaceae family. Previous studies have shown that terpenes and phenolics extracted from *Gardenia* sp. showed numerous potential bioactivities. In this study, the chemical constituents of *G. angkorensis*, their cytotoxic and *α*-glucosidase inhibition activities were investigated. The phytochemical investigation of the dichloromethane extract of *G. angkorensis* leaves growing in Vietnam has led to the isolation of 19*α*-hydroxyoleanolic acid 3-*O*-*β*-D-glucuronopyranoside (1), chikusetsusaponin IVa, 3 *β*, 16 *β*, 21 *β*, 23, 24-pentahydroxy urs-12,18,20-trien-28-oic acid-*γ*-lactone (3), linalool glucoside and linalyl 6-*O*-*α*-*L*-arabinopyranosyl-*β*-*D*-glucopyranoside (5). The identities of the compounds were based on the analysis of their NMR data. Compound 1 showed moderate inhibition against *α*-glucosidase with IC_{50} value of 58.01 ± 4.45 µM while compounds 3 (IC_{50} 186.60 ± 4.68 µM) and 5 (IC_{50} 239.53 ± 7.22 µM) exhibited weak cytotoxicity against the KB cell line and Hep G2 cell line (IC_{50} values 180.80 ± 7.16 µM, 253.95 ± 8.14 µM).

Keywords: *Gardenia angkorensis*, *α*-glucosidase inhibition, KB, Hep G2 cell line, cytotoxicity, triterpenes

Introduction

Gardenia is a large genus of the Rubiaceae family, which includes approximately 140 species distributed in tropical and subtropical regions of Africa, Southern Asia and Oceania. *Gardenia* species are found in several ecological environments, from primary forests to savannahs. Some species grow in swamps or mangroves. Numerous members of this genus are trees or shrubs. The difference in their growing environments is one of the reasons for the diversity of phytochemicals extracted from *Gardenia* species. Martins and Nunez (2015) showed that flavonoids, iridoids, iridoid glycosides, and other terpenoids are the main classes of compounds extracted from *Gardenia* species.[1] Previous studies revealed that phytochemicals of *Gardenia* sp. showed potential applications in human disease treatment and prevention. Genipin and geniposide from *G. jasminoides* could be applied in diabetes, while, crocetin expresses anti-oxidative stress and neurodegenerative prevention.[2,3] Sootepin A, sootepin B, coronalolide, coronalolide methyl ester, and tubiferolide methyl ester from *G. sootepensis* showed high cytotoxicity on several cancer cell lines including BT474 (breast cancer), CHAGO (lung cancer) with IC_{50} values from 1.8 to 6.8 µM.[4,5] Apart from studies on some species such as *G. jasminoides*, *G. sootenpensis*, and *G. lucida*, the phytochemical knowledge about this genus is still limited.

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The previous investigation on the water extract of *G. angkorensis* leaves led to the isolation of 14 compounds including two novel phenolic glycosides identified as angkorenside A and B.[6] These compounds showed moderate inhibition on nitric oxide production of lipopolysaccharide-activated murine macrophage RAW 264.7 cells. With the aim of the search for the bioactive compounds in *Gardenia* species, this follow-up study on the leaves of *G. angkorensis* resulted in the isolation of five compounds which were evaluated for *α*-glucosidase inhibition and cancer cell cytotoxicity.

Materials and Methods

Plant materials

Leaves of *Gardenia angkorensis* were collected at Binh Chau-Phuoc Buu Nature Reserve, Xuyen Moc district, Ba Ria-Vung Tau province, Vietnam in May 2020. The sample was identified by Le Van Son, Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology (VAST). A voucher specimen (ID: NF104.01-2019.329-2) was preserved at the Center for High Technology Research and Development, VAST.

General experimental procedures

^{1}H-NMR and ^{13}C-NMR, HSQC, COSY, and HMBC spectra were recorded on a Bruker Ascend 600 MHz spectrophotometer (Bruker, Corp) as follows: samples were dissolved in about 100 *µ*L of MeOD and transferred to Wilmad ® Micro NMR sample tubes. The spectra were recorded with a 1.7 mm TXI-probe head (Bruker, Corp) at the Institute of Chemistry, VAST. A small volume of sample was injected into Agilent 1100 single quadrupole LCMS system for mass spectrometry analysis. Tetramethylsilane (TMS) was used as an internal reference for chemical shifts and coupling constants (*J*) are given in Hertz (Hz). Preparative HPLC was performed on an Agilent 1200 HPLC system with YMC-Pack ODS-AQ column. Column chromatography (CC) was performed on silica gel 100 (63-200 µm) and C18 reversed-phase silica gel (RP-18, 15-25 µm), obtained from Merck Vietnam Ltd. Solvents were obtained from Samchun (Samchun

Chemical Co., Ltd., South Korea) and Merck Vietnam Ltd. TLC plates were visualized with 10% sulfuric acid followed by heating.

Extraction and isolation

The air-dried leaves (8.1 kg) were extracted twice with 15L of methanol 95% at room temperature by maceration combined with ultrasound extraction. The extract was filtered and concentrated in a vacuum evaporator to obtain MeOH extract (500 g) which was further dispersed in H_2O and successively partitioned with dichloromethane (DCM) and ethyl acetate (EtOAc).

The DCM extract (60 g) was subjected to a silica gel column (Ø80 mm x 800 mm, loaded with 250 g silica gel) and eluted with n-hexane and DCM-MeOH gradient (30:1 to 1:1) to yield three fractions (Fr.1-Fr.3) mediated through TLC. Fr.3 (7.0 g) was chromatographed on reverse-phase silica gel (Ø40 mm x 400 mm, loaded with 140 g C18 silica gel) with acetone: water as eluent system (2:1) to yield four subfractions from Fr.3.1 to Fr.3.4. Subfraction Fr.3.1 (1.29 g) was further fractionated on silica gel (Ø40 mm x 400 mm, loaded with 50 g silica gel) with DCM/MeOH (7:1) eluent system, yielding five more subfractions Fr.3.1.1 to Fr.3.1.5. Compound 1 (1.5 mg, 30% ACN, 0.5 mL/ min, T_R 24.4 min); 2 (5.8 mg, 60% MeOH, 0.5 mL/ min, T_R 54.5 min) and 5 (2.3 mg, 80% MeOH, 0.5 mL/ min, T_R 22.8 min) were obtained from Fr.3.1.2 (106 mg), Fr.3.1.4 (217 mg) and Fr.3.1.5 (125 mg), respectively by using preparative HPLC. Subfraction Fr.3.1.3 was further separated on a silica gel column with eluent system DCM/acetone/water (1/3.5/0.4) to yield compound 3 (1.1 mg) and compound 4 (2.1 mg, 23% ACN, 0.5 mL/ min, TR 38.4 min) by preparative HPLC.

Characterization of isolated compounds

19α-hydroxyoleanolic acid 3-*O*-*β*-*D*-glucuronopyranoside (1): amorphous powder. ESI-MS (m/z): 649.1 $[M+H]^+$ (Supplementary data, Figure S1). ^{1}H-NMR (600 MHz, CD_3OD) δ (ppm): 5.32 (t, *J*= 3.6 Hz, H-12), 3.27 (brd, *J*= 2.4, H-19), 3.22 (dd, *J*= 4.2, 12.0 Hz, H-3), 1.76* (m, H-21a), 1.29 (s, 3H-27), 1.07 (s, 3H-23), 1.03* (m, H-21b), 1.02 (s, 3H-30), 0.95 (s, 3H-25), 0.94 (s, 3H-29), 0.87 (s, 3H-24), 0.82 (s, 3H-26); *β*-D-glucuronopyranosyl: 4.36 (d, *J*= 7.8 Hz, H-1′), 3.56 (d, *J*= 9.6 Hz, H-5′), 3.45 (t, *J*= 9.6 Hz, H-4′), 3.39 (d, *J*= 9.0 Hz, H-3′), 3.25 (d, *J*= 7.8 Hz, H-2′) (Supplementary data, Figure S2). ^{13}C-NMR (150 MHz, CD_3OD) δ (ppm): 180.6 (C-28),145.15 (C-13), 124.61 (C-12), 90.78 (C-3), 82.97 (C-19), 57.16 (C-5), 49.62 (C-9), 47.26 (C-17), 45.70 (C-18), 42.62 (C-14), 40.76 (C-8), 40.21 (C-4), 39.66 (C-1), 38.06 (C-10), 36.04 (C-20),34.36 (C-7), 34.10 (C-22), 29.83 (C-16), 29.66 (C-21), 28.94 (C-15), 28.82 (C-29), 28.52 (C-23), 26.86 (C-2), 25.35 (C-30), 25.08 (C-27), 24.85 (C-11), 19.49 (C-6), 18.03 (C-26), 16.94 (C-24), 15.82 (C-25); *β*-D-glucuronopyranosyl: 176.5 (C-6′), 106.73 (C-1′), 78.05 (C-3′), 76.56 (C-5′), 75.59 (C-2′), 73.78 (C-4′) (Supplementary data, Figure S3). *Overlapping signals.

Chikusetsusaponin IVa (2): white powder. ESI-MS (m/z): 795.1 $[M+H]^+$ (Supplementary data, Figure S6). ^{1}H-NMR (600 MHz, CD_3OD) δ (ppm): 5.27 (m, H-12), 3.24 (m, H-3), 1.72 (m, H-19a), 1.41* (m, H-21a), 1.24* (m, H-21b), 1.18 (m, H-19b), 1.17 (s, 3H-27),1.06 (s, 3H-23), 0.96 (s, 3H-25), 0.95 (s, 3H-30), 0.93 (s, 3H-29), 0.86 (s, 3H-24), 0.82 (s, 3H-26); *β*-*D*-glucuronopyranosyl: 4.35 (d, *J*= 7.8 Hz, H-1′), 3.55 (d, *J*= 9.6 Hz, H-5′), 3.45 (m, H-4′), 3.39 (d, *J*= 9.0 Hz, H-3′), 3.25 (d, *J*= 7.8 Hz, H-2′); *β*-*D*-glucopyranosyl: 5,40 (d, *J*= 8.4 Hz, H-1″), 3.84 (m, H-6″a), 3.70 (m, H-6″b), 3.42 (m, H-3″), 3.39 (m, H-4″), 3.37 (m, H-2″), 3.37* (m, H-5″) (Supplementary data, Figure S7). ^{13}C-NMR (150 MHz, CD_3OD) δ (ppm): 176.99 (C-28), 144.79 (C-13), 123.92 (C-12), 90.59 (C-3), 57.04 (C-5), 49.02 (C-9), 48.06 (C-17), 47.21 (C-19), 42.94 (C-14), 42.32 (C-18), 40.75 (C-8), 40.20 (C-4), 39.87 (C-1), 37.89 (C-10), 34.93 (C-21), 34.00 (C-7), 33.47 (C-29), 33.19 (C-22), 31.53 (C-20), 28.92 (C-15), 28.53 (C-23), 26.88 (C-2), 26.27 (C-27), 24.57 (C-11), 24.03 (C-16), 23.95 (C-30), 19.34 (C-6), 17.00 (C-24), 17.76 (C-26), 16.04 (C-25); *β*-*D*-glucuronopyranosyl: 177.13 (C-6′),106.72 (C-1′), 78.08 (C-3′), 76.57 (C-5′), 75.60 (C-2′), 73.82 (C-4′); *β*-*D*-glucopyranosyl: 95.74 (C-1″), 78.70 (C-5″), 78.35 (C-3″), 73.96 (C-2″), 71.17 (C-4″), 62.47 (C-6″) (Supplementary data, Figure S8). *Overlapping signals.

3 *β*, 16 *β*, 21 *β*, 23, 24-pentahydroxy urs-12,18,20-trien-28-oic acid- *γ* -lactone (3): light yellow amorphous powder. ESI-MS (m/z): 499.1 $[M]^+$, 500.0 $[M+H]^+$ (Supplementary data, Figure S11). ^{1}H-NMR (600 MHz, CD_3OD) δ (ppm): 5.40 (m, H-12), 5.33 (s, H-30a), 5.26 (s, H-30b), 5.12 (d, *J*= 5.4 Hz, H-21), 4.15 (d, *J*= 11.4 Hz, H-24a), 4.09 (d, *J*= 11.4 Hz, H-23a), 3.78 (dd, *J*= 4.8, 11.4 Hz, H-3), 3.70 (d, *J*= 11.4 Hz, H-23b), 3.62 (d, *J*= 12.0 Hz, H-24b), 2.61 (dd, *J*= 5.4, 11.4 Hz, H-22a), 1.92 (d, *J*= 10.8 Hz, H-22b), 1.88 (s, 3H-29), 1.11 (s, 3H-26), 1.05 (s, 3H-25), 1.02 (s, 3H-27) (Supplementary data, Figure S12). ^{13}C-NMR (150 MHz, CD_3OD) δ (ppm): 178.48 (C-28), 143.98 (C-19), 139.41 (C-18), 137.61 (C-13), 129.22 (C-20), 128.54 (C-12), 114.12 (C-30), 81.89 (C-21), 74.58 (C-3), 67.35 (C-16), 63.64 (C-24), 63.19 (C-23), 55.67 (C-17), 49.28 (C-9), 49.14 (C-5), 47.28 (C-4), 44.39 (C-14), 40.44 (C-8), 39.68 (C-1), 39.14 (C-15), 37.61 (C-10), 35.20 (C-22), 34.26 (C-7), 28.04 (C-2), 26.84 (C-27), 24.34 (C-11),19.54 (C-6), 17.09 (C-26), 16.91 (C-25), 14.32 (C-29) (Supplementary data, Figure S13).

Linalool glucoside (4): Colorless viscous oil. ESI-MS (m/z): 317.1 $[M+H]^+$ (Supplementary data, Figure S17). ^{1}H-NMR (600 MHz, CD_3OD) δ (ppm): 6.08 (dd, *J*=7.2, 10.8 Hz, H-2), 5.19 (dd, *J*= 1.2, 18.0Hz, H-1a), 5.15 (dd, *J*= 1.2, 10.8 Hz, H-1b), 5.11 (m, H-6), 2.05 (d, *J*= 5.4 Hz, H-5a), 2.03 (d, *J*= 6.0 Hz, H-5b), 1.66 (s, 3H-9), 1.62 (d, *J*= 4.2 Hz, 2H-4), 1.59 (s, 3H-8), 1.32 (s, 3H-10); *β*-*D*-glucopyranosyl: 4.35 (d, *J*= 7.8 Hz, H-1′), 3.79 (dd, *J*= 2.4, 12.0 Hz, H-6′a), 3.64 (dd, *J*= 5.4, 12.0 Hz, H-6′b), 3.31 (d, *J*= 9.0 Hz, H-3′), 3.28 (d, *J*= 8.4 Hz, H-4′), 3.16 (d, *J*= 8.4 Hz, H-2′), 3.13 (d, *J*= 8.4 Hz, H-5′) (Supplementary data, Figure S18).^{13}C-NMR (150 MHz, CD_3OD) δ (ppm): 144.49 (C-2), 132.14 (C-7), 125.77 (C-6), 114.89 (C-1), 81.39 (C-3), 41.62 (C-4), 25.83 (C-9), 23.64 (C-5), 23.42 (C-10), 17.73 (C-8); *β* -*D*-glucopyranosyl: 99.54 (C-1′), 78.26 (C-3′), 77.59 (C-5′), 75.08 (C-2′); 71.75 (C-4′), 62.85 (C-6′) (Supplementary data, Figure S19).

Linalyl 6-*O*-*α*-*L*-arabinopyranosyl-*β*-*D*-glucopyranoside (5): amorphous powder. ESI-MS (m/z): 449.2 $[M+H]^+$ (Supplementary data, Figure S22). ^{1}H-NMR (600 MHz, CD_3OD) δ (ppm): 6.08 (dd, *J*=6.6, 10.8 Hz, H-2), 5.22 (dd, *J*= 1.2, 12.0 Hz, H-1a), 5.19 (dd, *J*= 1.2, 6.0 Hz, H-1b), 5.12 (m, H-6), 2.06 (d, *J*= 6.6 Hz, H-5a), 2.05 (d, *J*= 7.2 Hz, H-5b), 1.61 (s, 3H-9), 1.64 (d, m, 2H-4), 1.68 (s, 3H-8), 1.34 (s, 3H-10); *β*-*D*-glucopyranosyl: 4.35 (d, *J*= 7.8 Hz, H-1′), 4.03 (dd, *J*= 2.4, 11.4, H-6′a), 3.72 (dd, *J*= 4.8,11.4, H-6′b), 3.62 (dd, *J*= 6.6, 8.4, H-4′), 3.35 (d, *J*= 7.8, H-3′), 3.30 (t, *J*= 1.8, 3.6, H-5′), 3.18 (dd, *J*= 8.4, 9.0, H-2′); *α*-*L*-arabinopyranosyl: 4.33 (d, *J*= 6.6, H-1″), 3.88 (dd, *J*= 4.2, 8,4, H-5″a), 3.82 (m, H-4″), 3.56 (t, *J*= 3.6, H-3″), 3.55 (dd, *J*= 1.8, 8.4, H-5″b), 3.37 (d, *J*= 9.0, H-2″) (Supplementary data, Figure S23). ^{13}C-NMR (150 MHz, CD_3OD) δ (ppm): 144.34 (C-2), 132.12 (C-7), 125.78 (C-6), 115.16 (C-1), 81.48 (C-3), 41.69 (C-4), 25.89 (C-8), 23.67 (C-5), 23.49 (C-10), 17.76 (C-9); *β*-*D*-glucopyranosyl: 99.29 (C-1′), 78.08 (C-3′), 76.37 (C-5′), 75.04 (C-2′), 72.31 (C-4′); 69.21 (C-6′); *α*-*L*-arabinopyranosyl: 104.80 (C-1″), 74.07 (C-3″), 71,65 (C-2″), 69.26 (C-4″), 66.26 (C-5″) (Supplementary data, Figure S24).

α-Glucosidase inhibition assay

α-glucosidase inhibition assay of isolated compounds 1-5 was carried out on 96-well plates following Hakamata W et al (2009) and Acarbose was used as a control.[7] Briefly, samples were diluted with DMSO and deionized water to achieve the respective concentrations in mixtures at 10, 50, 200, and 250 µM. The reagents include phosphate buffer 100 mM pH 6.8; *α*-glucosidase 0.2 U/ml (G5003, Sigma-Aldrich, Ilc), sample, and 2.5 mM *p*-nitrophenyl-*β*-D-glucopyranoside. In the control sample, the sample volume was replaced with the phosphate buffer. The experimental solutions were incubated at 37°C. After 30 minutes, the reactions were stopped using Na_2CO_3. The absorbance of the reaction mixture was determined on a BIOTEK instrument at a wavelength of 410 nm (A). The ability of the test sample to inhibit the enzyme *α*-glucosidase was determined by the formula: Inhibition (%) = 100 x [A(control) – A(sample)] / A(control). Half maximal inhibitory concentrations (IC_{50}) were calculated using Table curve software.

In vitro cytotoxicity assay

The *in vitro* cytotoxicity assay of isolated compounds against human epithelial carcinoma cells (KB) and hepatocellular carcinoma cells (Hep

G2) was performed using an MTT assay following previously described methods.[8] Ellipticine was used as control and IC_{50} values were calculated using table curve software.

Statistical analysis

In the bioactivity assays, IC_{50} are presented as the mean ± standard deviation of three replications. Statistical significance was evaluated by ANOVA single factor analysis (Excel 2019, Microsoft, Corp) with p-values were calculated at 95.0% confidence interval. A mean comparison was performed with Duncan multiple range test at the 95.0% confidence level by using Statgraphics Centurion 19 (Statgraphics Technologies, Inc).

Results and Discussion

Structure elucidation

The structures of the isolated compounds (1-5, Figure 1) were elucidated by spectroscopic methods including 1D and 2D NMR analysis as well as by comparison with literature.

Compound 1 was isolated as a white powder. The ^{1}H-NMR spectrum of 1 showed an olefinic proton at δ_H 5.32 (t, J = 3.6 Hz, H-12), an anomeric proton δ_H 4.36 (d, J = 7.8 Hz, H-1′) and four oxymethine protons from 3.20 to 3.50 ppm suggesting the presence of a beta configuration of a carbohydrate moiety in the structure. ^{1}H-NMR and HSQC spectra revealed 18 methylene protons with chemicals shift from 1.00 to 2.30 ppm; two oxymethine protons at δ_H 3.22 (dd, J = 4.2, 12.0 Hz, H-3) and δ_H 3.27 (*brd*, J = 2.4 Hz, H-19); seven singlet signals of methyl groups at δ_H 1.29 (s, 3H-27), 1.07 (s, 3H-23), 1.02 (s, 3H-30), 0.95 (s, 3H-25), 0.94 (s, 3H-29), 0.87 (s, 3H-24), 0.82 (s, 3H-26) ppm. ^{13}C-NMR, HSQC and HMBC spectroscopic data for compound 1 showed the presence of 36 carbon atoms including 30 aglycone carbons and six glucoside carbons. The aglycone part exhibited specific features of an oleanolic acid triterpene with seven methyl signals at δ_C 28.8 (C-29), 28.5 (C-23), 25.4 (C-30), 25.1 (C-27), 18.0 (C-26), 16.9 (C-24), 15.8 (C-25); one carbonyl at δ_C 180.6 (C-28). The olefinic proton at δ_H 5.32 (t, J = 3.6 Hz, H-12) and two sp^2 carbon atoms at δ_C 124.6 (C-12), 145.2 (C-13) revealed a double bond between C-12 and C-13 of the oleanane skeleton. Chemical shift at δ_C 90.8 (C-3) and δ_H 3.22 (dd, J= 4.2, 12.0 Hz, H-3,) showed a *β*-OH at C-3. Meanwhile, the attachment of *α*-OH to C-19 was confirmed by the presence of δ_C 82.97 (C-19) and δ_H 3.27 (brd, J= 2.4 Hz, H-19) signals. Compared with previous reports, the aglycone part of compound 1 was identified as 19*α*-hydroxyoleanolic acid. The correlation signal between proton δ_H 3.56 (d, J = 9.6 Hz, H-5′) and the carbonyl at δ_C 176.5 (C-6′) in the compound's HMBC demonstrated the glycoside unit was *β*-D-glucuronopyranosyl. Besides, the cross peak between anomeric proton δ_H 4.36 (d, J = 7.8 Hz, H-1′) and δ_C 90.8 (C-3) provided evidence for the attachment of *β*-D-glucuronopyranose to the aglycone via O-*β*-glycoside linkage at C-3 position (Figure 2, Supplementary data, Figure S5). From these findings and comparing with literature data, compound 1 was identified to be 19*α*-hydroxyoleanolic acid 3-*O*-*β*-*D*-glucuronopyranoside[9] (Supplementary data, Table S1).

Compound 2 was isolated as a white powder. ^{1}H-NMR and HSQC showed signals for 20 methylene protons in the range 1.00 – 2.30 ppm and seven methyl protons at δ_H 1.06 (s, H-23), 0.86 (s, H-24), 0.96 (s, H-25), 0.82 (s, H-26), 1.17 (s, H-27), 0.93 (s, H-29), 0.95 (s, H-30). Two anomeric protons δ_H 4.35 (d, J = 7.8 Hz, H-1′) and δ_H 5.40 (d, J =8.4 Hz, H-1″) together with oxymethine protons from 3.20 to 3.80 ppm confirmed the relative configuration of monosaccharides in the structure of compound 2. The ^{1}H-NMR spectrum also showed one olefinic proton with chemical shift at δ_H 5.27 (m, H-12). ^{13}C-NMR, HSQC and HMBC spectra of compound 3 revealed 42 carbons of which 30 belonged to the aglycone and 12 to glycosides. Spectroscopic data indicated the similarity between the aglycone part of compounds 2 and 1, an oleanolic acid triterpene. Chemical shifts δ_C 106.7 (C-1′), 75.6 (C-2′), 78.1 (C-3′), 73.8 (C-4′), 76.6 (C-5′) and the correlation between proton δ_H 3,55 (m, H-5′) and carbonyl signal δ_C 177.1 (C-6′) confirmed the existence of a *β*-D-glucuronopyranosyl moiety. In comparison with previous reports, the chemical shifts at δ_C 95.7 (C-1″), 74.0 (C-2″), 78.4(C-3″), 71.2 (C-4″), 78.7 (C-5″), 62.5 (C-6″) belonged to a *β*-*D*-glucopyranosyl moiety. The positions of the two glycosides were determined based on analysis of its HMBC spectrum. The correlation signal between the anomeric proton δ_H 5.40 (d, J= 8.4 Hz, H-1″) and δ_C 176.99 (C-28) confirmed the attachment of *β*-glucopyranosyl to aglycone at position C-28. Meanwhile, *β*-D-glucuronopyranosyl attached to the oleanolic acid skeleton at C-3 position through O-glycoside linkage, which was confirmed by a cross peak between the anomeric proton at δ_H 4.35 (d, J = 7.8 Hz, H-1′) and δ_C 90.6 (C-3) (Figure 2, Supplementary data, Figure S10). From these observations and compared with literature, compound 2 was characterized to be chikusetsusaponin IVa[10] (Supplementary data, Table S2).

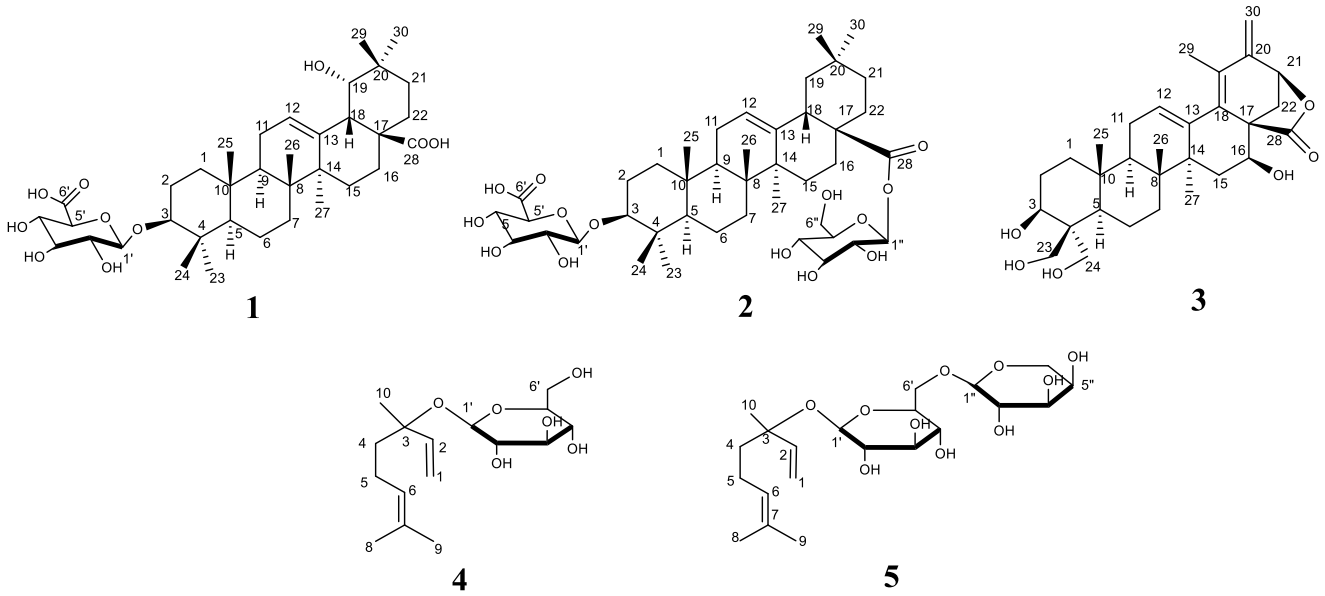

Figure 1: Structures of compounds 1-5

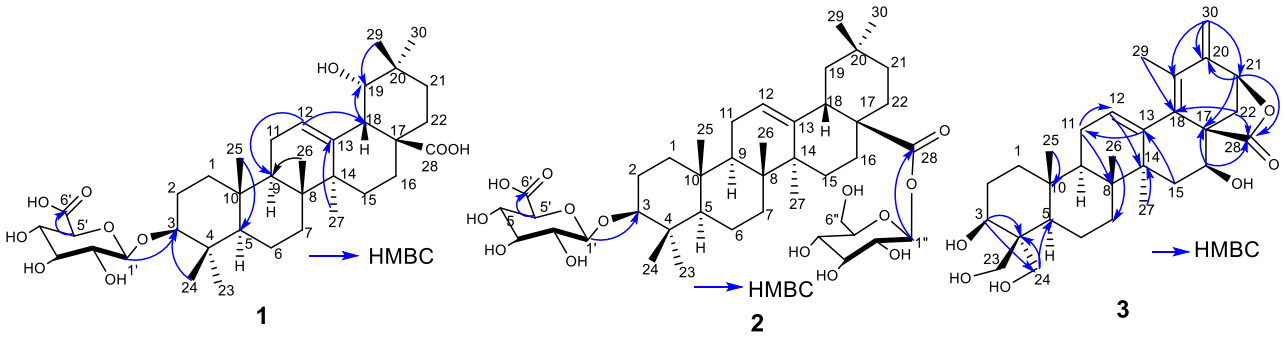

Figure 2: Key HMBC and COSY correlations of compounds 1-3

Compound 3 was isolated as a light-yellow amorphous powder. The 1D and 2D NMR of 3 showed an ursane-type triterpene with four methyl groups at δ_H 1.05 (s, H-25); 1.11 (s, H-26), 1.02 (s, H-27), 1.88 (s, H-29). The ^{13}C-NMR, HSQC, and HMBC spectra indicated the compound 3 had 30 carbon atoms including 1 carbonyl carbon at δ_C 178.48 (C-28) and four methyl carbons at δ_C 26,84 (C-27); 17,09 (C-26); 16,91 (C-25); 14,32 (C-29). Correlations between the methyl proton δ_H 1,88 (s, H-29) and δ_H 143,98 (C-19); exomethylene protons δ_H 5,33 (s, H-30a); δ_H 5,26 (s, H-30b) and δ_H 129.2 (C-20) confirmed an ursane skeleton for compound 3. Four methylene protons with chemical shifts between 3.60-4.30 ppm suggested the presence of two hydroxymethyl groups. Cross peak signals between δ_C 47.28 (C-4) and two protons δ_H 4,09 (d, J = 11.4 Hz, H-23a); δ_H 3,62 (d, J = 12,0 Hz, H-24b) indicated these hydroxymethyl groups were attached to ring A at position C-4. Proton δ_H 5,40 (m, H-12) and two sp^2 methylene carbons δ_C 128,54 (C-12); 137,61(C-13) were assigned for C-12/C1-3 double bond of ursane skeleton. Proton δ_H 3,78 (dd, J= 4,8, 11,4 Hz, H-3) and δ_C 74,58 (C-3) confirmed the *β*-OH at C-3 of ursane skeleton. COSY and HMBC spectra also showed an *α*-OH at C-16 (δ_C 67.35) position. On the other hand, the correlations between δ_H 5,12 (d, J= 5.4 Hz, H-21); 1,92 (d, J= 10.8 Hz, H-22) and carbonyl carbon δ_C 178,48 (C-28) showed a *γ*-lactone at C-28 (Figure 2, Supplementary data, Figure S14, S16). Compared with previous reports, compound 3 was identified to be 3*β*, 16*β*, 21*β*, 23,24-pentahydroxy urs-12,18,20-trien-28-oic acid-*γ*-lactone[11] (Supplementary data, Table S3).

Compound 4 was isolated as a colorless viscous oil. The spectroscopic data of compound 4 showed characteristics of a monoterpene glycoside. The ^{13}C-NMR, HSQC, and HMBC spectra of compound 4 confirmed the presence of 16 carbon atoms. The aglycone part with 10 carbons was identified to be lynalool, in which δ_C 114.89 (C-1), δ_C 144.49 (C-2) belong to C-1/C-2 double bond, δ_C 125.77 (C-6) and δ_C132.14 (C-7) were assigned to C-6/C-7 double bond. Correlation signals between δ_H 1.32 (s, 3H-10) and δ_C 81.39 (C-3) on HMBC confirmed the attached of methyl group δ_C 23.42 (C-10) at C-3 position. Similarly, two methyl groups δ_C 17.73 (C-8) and δ_C 25.83 (C-9) were at C-7 position. Anomeric proton 4.35 (d, J= 7.8 Hz, H-1´) together with large coupling constant of oxymethin protons in range 3.0 to 3.4 ppm showed the presence of a *β*-D-glucopyranosyl moiety as compared with previous publications. This glycosyl moiety attach to the lynalool at C-3 position, which was confirmed by the cross signal between δ_H 4.35 (d, J= 7.8 Hz, H-1′) and δ_C 81.48 (C-3) on HMBC (Supplementary data, Figure S17-21, Table S4). Compared with previous reports, compound 4 was identified to be linalool glucoside[12]

Compound 5 was isolated as amorphous powder. The spectroscopic data of compound 5 showed similarities with compound 4, which is a lynalool glycoside. However, the glycoside part was determined to be a disaccharide, 6-*O*-*α*-L-arabinopyranosyl-*β*-*D*-glucopyranosyl moiety based on the previous data and signal between anomeric proton δ_H 4.33 (d, J= 6,6 Hz, H-1″) and δ_C 69.21 (C-6′) on HMBC. Cross peak between δ_H 4.35 (d, J= 7.8 Hz, H-1′) and δ_C 81.48 (C-3) confirmed the attached of disaccharide moiety to aglycone ats C-3 position through *O*-glycoside linkage (Supplementary data, Figure S22- 26, Table S5). From these observations and compared with previous publishes, compound 5 was characterized to be linalyl 6-*O*-*α*-L-arabinopyranosyl-*β*-D-glucopyranoside[13]

α-glucosidase inhibition assay

All isolated compounds 1-5 were examined for the inhibition against *α*-glucosidase enzyme to evaluate their application in the treatment of diabetes. The results showed that amongst tested compounds only 19*α*-hydroxyoleanolic acid 3-*O*-*β*-*D*-glucuronopyranoside (1) expressed noticeable inhibition activities with IC_{50} values of 58.01 ± 4.45 µM and linalyl 6-*O*-*α*-*L*-arabinopyranosyl-*β*-*D*-glucopyranoside (5) showed weak inhibition with IC_{50} 265.93 ± 20.46 µM (Figure 3, Table 1). Meanwhile, Acarbose, an FDA-officially approved drug for diabetic treatment, had an IC_{50} value of about 242.68 ± 0.01 µM. These results could provide evidence for the potential application of 19*α*-hydroxyoleanolic acid 3-*O*-*β*-*D*-glucuronopyranoside (1) in diabetic treatment.

Table 1: α-glucosidase inhibition and cytotoxicity of isolated compounds 1-5

Compounds	IC_{50} (µM)		
	***α*-glucosidase**	**KB**	**Hep G2**
1	58.01^{a*} ± 4.45	I	I
2	I	I	I
3	I	186.60^{a} ± 4.68	180.80^{a} ± 7.16
4	I	I	I
5	265.93^{b} ± 20.46	239.53^{b} ± 7.22	253.95^{b} ± 8.14
Acarbose	242.68^{b} ± 0.01	-	-
Ellipticine	-	1.66^{c} ± 0.08	1.71^{c} ± 0.08

I: Inactive, * Mean ± SD values in the same column with different superscript letters are significantly different at the 95.0% confidence level. Acarbose and Ellipticine as positive controls for *α*-glucosidase inhibition and cytotoxicity assays, respectively

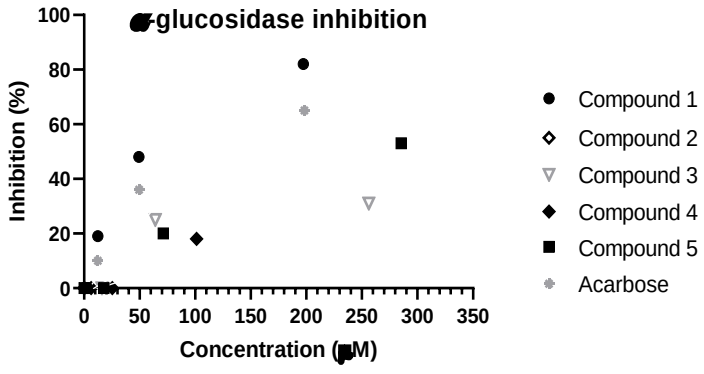

Figure 3: *α*-glucosidase inhibition assay of compounds 1-5

Cancer cell cytotoxicity

Purified compounds 1-5 were evaluated for the cytotoxicity on human epithelial carcinoma cells (KB), and hepatocellular carcinoma cells (Hep G2). The results showed that compounds 3 and 5 expressed weak activity on KB and Hep G2 cell lines with IC_{50} values varied from 180.80 ± 7.16 to 253.95 ± 8.14 µM (Table 1). The toxicities were relatively lower compared to several previously investigated terpene compounds. [14] However, compound 3 was more toxic on Hep G2 than friedelin, a triterpene with the same pentacyclic structure.[15]

Conclusion

In the present study, five compounds including 19*α*-hydroxyoleanolic acid 3- *O*-*β*-D-glucuronopyranoside (1), chikusetsusaponin IVa (2), 3*β*, 16*β*, 21*β*, 23, 24-pentahydroxy urs-12,18,20-trien-28-oic acid-*γ*-lactone (3), linalool glucoside (4) and linalyl 6-*O*-*α*-*L*-arabinopyranosyl-*β*-*D*-glucopyranoside (5) were isolated from a dichloromethane fraction of *Gardenia angkorensis* leaves. Bioactivity evaluation revealed the potential inhibition by 19*α*-hydroxyoleanolic acid 3-*O*-*β*-*D*-glucuronopyranoside (1) of *α*-glucosidase with IC_{50} of 58.01 ± 4.45 µM. On the other hand, only 3*β*, 16*β*, 21*β*, 23, 24-pentahydroxy urs-12,18,20-trien-28-oic acid-*γ*-lactone (3) and linalyl 6-*O*-*α*-*L*-arabinopyranosyl-*β*-*D*-glucopyranoside (5) showed weak cytotoxic effects against the KB cell line (IC_{50} values 186.60 ± 4.68, 239.53 ± 7.22, respectively) and Hep G2 cell line (IC_{50} values 180.80 ± 7.16, 253.95 ± 8.14, respectively).

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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