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Structure-Activity Relationship of Triterpenoids Isolated from *Mitragyna stipulosa* on Cytotoxicity

Leon Azefack Tapondjou[1], David Lontsi, Beiham Luc Sondengam[2], Muhammad Iqbal Choudhary[3], Hee-Juhn Park[4], Jongwon Choi[5], and Kyung-Tae Lee[6]

[1]Department of Chemistry, Faculty of Science, University of Dschang, box, 183, Dschang, Cameroon, [2]Department of Organic Chemistry, Faculty of Science, University of Yaounde I, Box 812, Yaounde, Cameroon, [3] International Center for Chemical Sciences, HEJ Research Institute of Chemistry, University of Karachi, Karachi 75270, Pakistan, [4]Division of Applied Plant Sciences, Sangji University, Wonju 220-702, Korea, [5]College of Pharmacy, Kyungsung University, Pusan 608-736, Korea, and [6]College of Pharmacy, Kyung Hee University, Seoul 130-701, Korea

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Chromatographic separation of the stem bark extract of *Mitragyna stipulosa* afforded triterpene derivatives ursolic acid (**1**), quinovic acid (**2**), quinivic acid 3-O-β-D-glucopyranoside (**3**, quinovin glycoside C), quinovic acid 3-O-[(2-O-sulfo)-β-D-quinovopyranoside] (**4**, zygophyloside D) and quinovic acid 3-O-β-D-quinovopyranosyl-27-O-β-D-glucopyranosyl ester (**5**, zygophyloside B). These five compounds were subjected to the cytotoxicity on MTT assay system. Compound **1** among tested showed the most potent cytotoxicity. Quinovic acid showed less potent cytotoxicity than ursolic acid and sugar linkages to **2** decreased the cytotoxicity. Compound **4** more potent than **3** with indicate that the sulfonyl group significantly enhances the activity. This indicates that the glycosidic linkage in ursane-type triterpenoids has mainly negative effect on cytotoxicity unlike in oleanane-type glycosides.

Key words: *Mitragyna stipulosa*, Triterpenoid, Ursane-type, Cytotoxicity, Structure-activity relationship

INTRODUCTION

Mitragyna stipulosa Korth. is a tree which grows to a height of about 3 to 4 m and is widely distributed through South East Asia and sub-tropical Africa. Although there are no records on the utility of this plant in the Camerounian traditional medicine, its stem bark have been used to treat neuralgia pain, cancer and diabetes by the local people of the southern Cameroon for a long time. It has been found that sugar linkage positions to triterpenes usually determine the cytotoxicity potency against cancer cell lines (Park *et al.* 2001; Lee *et al.* 1998; Kim *et al.* 2000). In the course of searching for cytotoxic triterpenoids, we isolated triterpenoids from *M. stipulosa* and tested cytotoxicity by MTT method. By these experiments a significant structure-cytotoxicity relationship on ursane-type triterpenoids was revealed. A genuine structure of ursane-type triterpenoids for the cytotoxicity was attributed to the aglycone rather than their glycoside.

Correspondence to: Hee-Juhn Park, Division of Applied Plant Science, Sangji University, 660, Woosan-dong, Wonju 220-702, Korea
E-mail: hjpark@mail.sangji.ac.kr

MATERIALS AND METHODS

General experimental procedures

Melting points were determined by using the Yanaco micro melting point apparatus. IR spectra were recorded on the Jasco A-302 spectrophotometer. The ^{1}H-NMR spectra (δ ppm, *J* in Hz) were recorded in deuterated solvents on a Bruker AM-300 or AM-400 spectrometer (300 or 400 MHz) while ^{13}C-NMR spectra were recorded in the same solvent on an AM-400 instrument at 100 MHz with tetramethylsilane (TMS) as an internal standard. EI-MS and FAB-MS were taken on the mass spectrometer MAT 311A and the mass spectrometer JMS HX-110, respectively. The purity of the samples was checked on TLC (Si gel, precoated plates, Merck, PF_{254}, 20 × 20, 0.25 mm). Solvents were distilled prior to use.

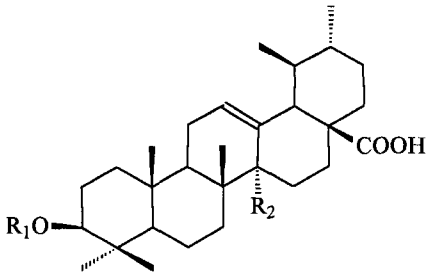
Plant material

The stem bark of this plant were collected in Mfou near Yaoundé, Cameroon in February 1998 and were identified by the botanists of the Department of Plant Biology of the University of Yaounde I, Cameroon. Specimens documenting the collection are deposited at the Cameroon National Herbarium (ref: 21076/SRF/CAM).

Extraction and isolation of compounds

The dried, pulverized bark of *M. stipulosa* (3 kg) were extracted in the soxhlet extractor with 95% EtOH to give an ethanolic extract (180 g). A part of this extract (150 g) was dissolved in 1.0 L of 3%-aqueous HCl and filtered. The filtrate was adjusted to pH 10-11 by 5%-NaOH and extracted with $CHCl_3$ to obtain an alkaloidal fraction (20 g). The acid soluble fraction was neutralized with water and extracted successively with ethyl acetate and n-BuOH. The ethyl acetate fraction (25 g) was subjected to silica gel column chromatography with a gradient of n-hexane-EtOAc (9:1 to 5:5) each 2000 ml. One subfraction were purified by silica gel column chromatography with a gradient of hexane-EtOAc (9.5:0.5 to 7:3) to afford α-amyrin, 3-O-acetyl ursolic acid and a mixture of phytosterols. The other subfraction was also subjected to silica gel column chromatography to afford ursolic acid (1, 320 mg) and quinovic acid (2, 4 g) together with the β-sitosterol β-D-glucopyranoside (400 mg).

The n-BuOH layer was evaporated to dryness under reduced pressure to yield 120 g of crude extract. A part the above extract (70 g) was subjected to silica gel column chromatography and eluted with a mixture of CH_2Cl_2-MeOH with increasing polarity of MeOH to afford 3 main fractions A, B and C. The fraction A (5 g) was rechromatographed on silica gel (300 g) with a gradient of $CHCl_3$-MeOH (8:2 to 6:4) to give compound 2 in pure form (300 mg) and a mixture mainly consisted of compound 3 and 4. This mixture (2.5 g) was subjected to repeated chromatography in silica gel column chromatography (150 g) eluted with a continuous gradient of $CHCl_3$-MeOH (7:3) to afford compound 3 (800 mg) and an impure fraction mainly consisted of compound 4 whose purification has been achieved by further recrystallisation in MeOH. The second fraction B (3 g) was rechromatographed on a silica gel gel column chromatography (250 g) and eluted with the mixture of $CHCl_3$-MeOH (7:3 to 5:5) to afford two subfractions B1 and B2. Fraction B1 upon purification by recrystalisation in the mixture $CHCl_3$-MeOH-AcOH-H_2O (6:4 0.25:0.25) mainly afforded compound 5 (700 mg).



1. R_1=R_2=H (ursolic acid)
2. R_1=H, R_2=COOH (quinovic acid)
3. R_1=β-D-glc(*p*), (quinovin glycoside C)
4. R_1=(2-O-sulfo)-β-D-qui(*p*), R2=COOH (zygophyloside D)
5. R_1-β-D-qui(*p*), R2=β-D-glc(*p*) (zygophyloside B)

Fig. 1. Structure of triterpene glycosides (**1-5**) isolated from *M. stipulosa*.

Compound 1 (Ursolic acid): Colorless needles, mp 282-283°; ^{1}H-NMR(C_5D_5N, 300MHz): δ=5.48 (1H, t, *J*=3.4 Hz H-12), 3.45 (1H, t, *J*=6.9 Hz, H-3), 2.62 (1H, d, *J*=11.0 Hz, H-18 Hz), 1.24 (6H, s, 2 × CH_3), 0.96 (3H, d, *J*=5.7 Hz, CH_3), 1.06, 1.02, 1.01, 0.90 (each 3H, s, CH_3); ^{13}C-NMR(C_5D_5N, 75 MHz) δ: Table 1.

Compound 2 (Quinovic acid): amorphus white powder, MP: 300°C, IR (KBr) ν_{max} (cm^{-1}): 2925, 1705, 1640, 1460. EIMS: m/z 486 (M^+, $C_{30}H_{46}O_5$, calcd.), 486(M^+ -H_2O), 441(M^+-C OOH), 427, 380, 277, 207, 190. ^{1}H-NMR(C_5D_5N, 300MHz): δ=6.01 (1H, br s, H-12), 3.30 (1H, dd, *J*=5.0 Hz, *J*=10.6Hz, H-3), 2.80 (1H, d, *J*= 11.1 Hz, H-18), 1.20 (3H, d, *J*= 5.9 Hz, CH_3-29), 1.12, 1.05, 0.97, 0.91 (3H each, s, H-23,-24,-25,-26), 0.79 (3H, d , *J*=6.0 Hz, H-30). ^{13}C-NMR(C_5D_5N, 75 MHz) δ: Table 1.

Compound 3 (Quinovic acid 3-O-β-D-glucopyranoside, Quinovin glycoside C): White powder, mp 270-272°C, IR (KBr) ν_{max} (cm^{-1}): 3480, 2980, 1710, 1680, 1450, 1080. Mass (FAB negative ion): m/z 647 $[M-H]^-$ ($C_{36}H_{56}O_{10}$, calcd.), 603 $[M-H-CO_2]^-$,485 $[M-H-Glc]^-$; ^{1}H-NMR (C_5D_5N, 400MHz): δ=6.0 (1H, br s, H-12), 4.7 (1H, d, *J*=7.7Hz, H-1 of Glc), 3.20 (1H, dd, *J*=2.6 Hz, J=11.6Hz, H-3), 2.80 (1H, d, *J*=11.2Hz, H-18), 1.20 (3H, d, *J*= 4.9 Hz, H-29), 1.12, 1.07, 0.93, 0.84 (3H each, s, H-23,-24,-25,-26), 0.79 (3H, d , *J*=4.2 Hz, H-30).

Compound 4 (zygophyloside D): White powder, $[\alpha]_D$ +40.9° (C=1.0, CH_3OH), IR (KBr) ν_{max} (cm^{-1}): 3423 (OH), 2929 (C-H), 1693 (COOH), 1457, 1386, 1283 (C-O), 1070 (glycosidic C-O); ^{1}H-NMR(CD_3OD, 400MHz): δ=0.84 (3H, s, H-23), 0.89 (3H, s, H-26), 0.92 (3H×2, d, J=7.67, H-29 & 30), 0.96 (3H, s, H-25), 1.04 (3H, s, H-24), 1.27 (3H, d, *J*=6.04 Hz, H-6 of Qui), 3.00 (1H, m, H-3 of Qui), 3.09 (1H, m, H-5 of Qui), 3.33 (1H, m, H-5 of Qui), 3.60 (1H, t, *J*=9.04 Hz, H-3 of Qui), 4.05 (1H, t, *J*=7.72 Hz, H-2 of

Qui), 4.43 (1H, d, *J*=7.72 Hz, H-1 of Qui), 5.59 (1H, m, H-12; Mass (FAB negative ion): m/z 711 $[M-H]^-$ ($C_{36}H_{56}O_{12}S$, calcd.).

Compound 5 (Quinovic acid 3-O-β-D-quinovopyranosyl-27-O-β-D-glucopyranosyl ester, zygophyloside B): White powder, mp 218-220°C, IR (KBr) ν_{max} (cm^{-1}): 3490, 2985, 1720, 1690, 1460,. Mass (FAB negative ion): m/z 793 $[M-H]^-$, ($C_{42}H_{66}O_{14}$, calcd.), 587 $[M\text{-}Qui\text{-}CO_2\text{-}O]^-$, 571 $[M\text{-}H\text{-}Glc\text{-}O\text{-}CO_2H]^-$ 425 $[587\text{-}Glc]^-$; ^{1}H-NMR(CD_3OD, 400MHz) δ=5.60 (1H, br s, H-12), 5.20 (1H, d, *J*=7.8Hz, H-1 Glc), 4.32 (1H, d, *J*=7.2Hz, H-1 of Qui), 2.72 (1H, d, *J*= 11.2Hz, H-18), 1.31 (3H, d, *J*= 5.9Hz, CH_3-Qui), 1.00, 0.95, 0.88, 0.82 (3H each,s, H-23,-24,-25,-26), 0.89 (3H, d, *J*=4.2Hz, CH_3-30). ^{13}C-NMR(C_5D_5N, 75MHz) δ: Table 1.

MTT assay for cell viability

Cytotoxicity studies were performed in a 96-well plate. RAW 264.7 cells were mechanically scraped and plated 2×10^5/well in 96-well plate containing 100 ml of DMEM medium with 10% FBS and incubated overnight. Hederagenin monodesmosides were dissolved in dimethylsulfoxide (DMSO) and the DMSO concentrations in all assays did not exceed 0.1%. Twenty-four hours after seeding, 100 ml new media or hederagenin monodesmosides was added, and the plates were incubated for 24 h. Cells were washed once before adding 50 ml FBS-free medium containing 5 mg/ml MTT. After 4 h of incubation at 37°C, the medium was discarded and the formazan blue, which formed in the cells, was dissolved in 50 ml DMSO. The optical density was measured at 540 nm. The concentration required to reduce absorbance by 50% (IC_{50}) in comparison to control cells was determined (Denizot et al., 1996).

RESULTS AND DISCUSSION

Silica gel column chromatography of the EtOAc fraction led to the isolation of compound **1** and **2**. Compound **1** was identified as ursolic acid by comparison of mp, $[\alpha]_D$ and NMR spectra of an authentic specimen (Park *et al.*, 1993). In the ^{1}H-NMR spectrum of compound **2**, six methyl signals and chrateristic ursane-type sgnals were observed at δ 1.20 (3H, d, *J*=5.9 Hz, H-29) and 0.79 (3H, d, *J*=5.9 Hz, H-30). The absorption band at 1705 cm^{-1} in the IR spectrum and the two peaks at both δ 177.7 (C-27) and 179.8 (C-28) in ^{13}C-NMR spectrum inducated that this compound has two carboxyl groups. ^{13}C-NMR data of **2** were in agreement with the reported ones of quinovic acid (Ahmad *et al.*, 1994).

IR spectroscopic data at 1080 cm^{-1} (glycoside) and FAB-MS data at m/z 647 $[M-H]^-$ indicated that compound

Table 1. ^{13}C-NMR signals (ppm) of triterpenoids (1-5) isolated from the *M. stipulosa* stem bark (75 MHz).

Carbon	1	2	3	4	5	Carbon	1	2	3	4	5
1	38.7	39.1	38.8	39.9	40.5	23	23.4	27.8	19.2	19.1	19.3
2	23.5	26.2	26.5	27.1	27.1	24	17.0	16.4	16.3	28.5	28.5
3	79.0	77.8	88.6	91.3	90.7	25	17.0	18.0	18.0	16.9	18.2
4	39.6	39.9	39.4	40.2	40.1	26	15.5	16.4	16.9	18.2	16.9
5	52.7	55.6	55.6	56.9	56.9	27	24.2	177.7	177.8	179.0	178.8
6	18.3	18.7	18.4	19.3	18.2	28	176.0	179.8	179.8	181.7	179.2
7	33.0	37.3	37.3	37.7	37.8	29	21.1	18.7	18.7	17.1	17.1
8	39.1	37.2	36.9	40.7	37.0	30	23.4	21.1	21.1	21.6	21.5
9	47.6	47.1	47.0	48.0	48.0	3-O-G					
10	36.7	37.2	36.9	37.9	38.0	1			106.6	104.1	106.3
11	23.7	23.2	23.1	23.9	23.9	2			75.5	82.0	75.9
12	125.8	128.8	128.8	130.4	130.9	3			78.5	77.5	78.0
13	138.0	133.9	133.9	133.9	133.3	4			71.8	76.8	77.0
14	42.0	56.7	56.9	57.3	57.4	5			77.6	72.6	73.0
15	29.4	25.3	25.3	25.8	25.8	6			62.9	18.1	18.2
16	23.3	26.2	26.2	26.5	26.4	27-O-G					
17	47.9	48.5	48.5	-	48.5	1					95.7
18	55.3	54.7	54.8	55.6	55.4	2					73.4
19	30.6	37.5	37.6	40.4	40.9	3					78.6
20	30.4	39.2	39.2	38.4	38.3	4					71.3
21	27.3	30.4	30.4	31.3	31.2	5					78.3
22	37.0	36.9	36.9	38.1	38.5	6					62.6
Solvent	$CDCl_3$	C_5D_5N	C_5D_5N	CD_3OD	C_5D_5N	Solvent	$CDCl_3$	C_5D_5N	C_5D_5N	CD_3OD	C_5D_5N

- masked

Table 2. Cytotoxic activities of 1-4 isolated from *Mitragyna stipulosa*

Treatment	IC_{50}[a] (g/ml)			
	HL-60	U-937	A549	3LL
1 (Ursolic acid)	22.5	31.8	42.6	31.3
2 (Quinovic acid)	46.0	81.5	24.0	88.9
3 (quinovin glycoside C)	>100	>100	>100	>100
4 (zygophyloside D)	62.6	20.4	76.4	>100
5 (zygophyloside B)	>100	>100	>100	>100
Cisplatin	8.6	26.1	21.6	19.8

[a] IC_{50} is defined as the concentration which resulted in a 50% decrease in cell number. The values reprsent the mean of three independent experiments.

3 is a triterpene glycoside. Its ^{1}H-NMR spectrum showed six methyl signals including the two doublet methyls at δ 1.20 (3H, d, *J*=4.9 Hz, H-29) and 0.79 (3H, d, *J*=4.9 Hz, H-30). As shown in Table 1, the ^{13}C-NMR data of **3** indicated that this compound has quinovic acid as an aglycone and D-glucose as a sugar moiety. Sugar linkage was found to be configurated as β since the anomeric proton at δ 4.70 was splitted as *J*=7.7 Hz. Therefore, **3** is quinovic acid 3-O-β-G-glucopyranoside (quinovin glycoside C) (Aquino *et al.*, 1989).

IR absorption bands at 1693 cm^{-1} (COOH) and 1070 cm^{-1} (glycoside) and other bands of compound **4** indicated that this compound is also a triterpene glycoside. In ^{1}H-NMR spectrum of 4, the two doublet peaks at 0.92 (3H×2, d, *J*=7.7 Hz, H-29 and 30) due to quinovic acid and another doublet at δ 1.27 (3H, d, *J*=6.04 Hz) of H-6 of D-quinovose. Additionally, the anomeric proton of D-quinovose were shown as doublet (*J*=7.7 Hz) at δ 4.43. The ^{13}C-NMR data of D-quinovose moiety was not in accordance with those of the other compounds which have D-quinovose moiety. FAB-MS data indicated that a sulfonyl is substituted to the parent skeleton. Spectroscopic data of 4 were in accordance with the reported data of zygophyloside (Quinovic acid 3-O-[(2-O-sulfo)-β-D-quinovopyranoside] (Ahmad *et al.*, 2000). The ^{13}C-NMR data were completely assigned to zygophyloside D aided by ^{1}H-^{1}H COSY-, ^{1}H-^{13}C COSY and HMBC NMR spectra.

In the IR spectrum of compound **5**, the absorption bands at 1720 cm^{-1} due to ester and at 1690 cm^{-1} due to carboxyl were observed. Four singlet methyl signals and two doublet (*J*=4.2 Hz) methyl signals due to H-29 and 30 implied that this compound is a quinovic acid derivative. Two anomeric protons at δ 5.20 (1H, d, *J*=7.8 Hz, Glc) and δ 4.30 (1H, d, *J*=7.2 Hz, Qui) indicated that 5 has two sugar units. In HMBC spectrum of **5**, δ_H 4.30 was correlated with δ_C 90.7 and a peak at δ_H 5.20 was crossed with δ_C 178.8. Therefore, compound **5** was identified as zygophyloside B (quinovic acid 3-O-β-D-quinovopyranosyl-27-O-β-D-glucopyranosyl ester) (Safir *et al.*, 1998).

On MTT assay, ursolic acid showed the most potent cytotoxicity whereas quinovic acid with 27-carboxyl to ursolic acid exhibited much less activity than **1**. Attachemt of D-glucose to quinovic acid was observed not to increase the cytotoxicity of quinovic acid from the cytotoxicity of **4**. Sulfonyl group substitution to C-2 of D-glucose in **4** led to a little higher activity than that of 3 itself, indicating that the sulfonyl group has activity-enhancing action. Sugar attachment at both sites of C-3 and C-27 completely blocked the cytotoxicity on compound **1** and **2**. Observation of non-cytotoxicity of **5** led to the finding that 28-carboxyl is not essential for the cytotoxicity but explained that a bisdesmoside based on hydrophilic-hydrophobic-hydrophilic structure may be critical for the non-cytotoxicity as usually shown by bisdesmoside form of saponins. The cytotoxicity result of **1-5** suggested that the aglycone of saponin with ursane-type sapogenin could be a true form for the cytotoxicity in plant kingdom. This hypothesis could be also supported by very rare natural occurrence of the saponins with both an ursane-type triterpene and an oligosaccharide.

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