REPORT

Structures and hepatocytotoxicity of co-occurring substances in oleanolic acid tablets

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Abstract: Tablets of oleanolic acid (OA) have been approved by SFDA in China as an adjuvant therapy for acute and chronic hepatitis. Co-occurring substances present in the tablets of OA and their hepatocytotoxicity have not yet been reported. In the current investigation, the crude OA drug was separated by repeated column chromatography. The structures of the isolated compounds were characterized by spectral analysis and the cytotoxicity of each compound was evaluated *in vitro* against the human normal liver cell L02 at concentrations from 0.125 to 1000 μmol/L using the MTT method. As a result, OA and its 11 co-occurring trace compounds including one new triterpenoid, 3-*O*- (4-oxo-pentanoyl)-olean-12- en-28-oic acid, were isolated and structurally characterized. Cytotoxicity tests indicated that these compounds were all non-toxic at concentrations up to 50μmol/L. Clear structure-activity relationship (SAR) was also observed. The results suggested that OA tablets of similar origin might not cause obvious cytotoxicity to the normal liver cell. The work may facilitate further SAR studies of OA-type triterpenoids.

Keywords: Co-occurring substances; pentacyclic triterpenoids; cytotoxicity, sapogenins.

INTRODUCTION

leanolic acid (OA) is a pentacyclic triterpenoid widely present in natural products either as a free acid or as an aglycone of triterpenoid saponins (Liu, 1995, 2005). It was reported that OA pretreatment dramatically diminished carbon tetrachloride-, bromobenzene-, acetaminophen-, phalloidin-, and cadmium-induced liver injury, and decreased the hepatotoxicity of D-galactosamine, endotoxin, thioacetamide, furosemide, and colchicine (Liu *et al*., 1995; Liu *et al*., 2008; Pollier and Goossens, 2012). Tablets of OA have been approved by SFDA in China as an adjuvant therapy for acute and chronic hepatitis.

High performance liquid chromatography (HPLC)-diode array detector (DAD) analysis indicated that quite a number of OA's co-occurring substances in trace amounts were detected in its various tablets. Although the total co-occurring substances (less than 5% of the crude drug) did not produce an obvious effect on the cytotoxicity of OA, the collective effect of the co-occurring substances should not be ignored if they have significant cytotoxicity. Understanding the structures and toxicities of these compounds was thus of special importance. Chemical investigations were therefore conducted for these minor compounds. As a result, OA and its 11 co-occurring trace compounds (two nortriterpenoids and nine triterpenoids) (fig. 1) including one new triterpenoid were isolated and structurally characterized from the crude drug of OA. Cytotoxicity of these sapogenins against the human normal liver cell line L02 was evaluated and clear structure-hepatocytotoxicity relationship was observed.

MATERIALS AND METHODS

General

Semi-preparative HPLC: Agilent-1100 MWD; Hedera-C18 column (ODS-2, 250 × 30.0 mm, 5μm). UV: Shimadzu UV-2401PC. Optical rotations: Rudolph Autopol α. IR: Bruker Vector-22. NMR: JEOL ECX-500 spectrometer with TMS as an internal standard. ESIMS: Waters Acquity UPLC-TQD. HRESIMS: microTOF-Q II. Solvents used for preparation were of analytical grade. Human hepatocyte cell line L02 was purchased from Shanghai Institute of Biochemistry and Cell Biology. RPMI 1640 culture medium was provided by Gibco Industries Inc. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was obtained from Sigma-Aldrich (Shanghai, PR China). Bovine serum was provided by Tianjin Haoyang Biological Manufacture Co. Ltd. (Tianjin, PR China). 0.25% Trypsin was provided by Merck Co. (Darmstadt, Germany).

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Crude drug

The crude drug of OA (25 kg/carton) with purity of *ca* 95% was bought from Sichuan Province Yuxin Pharmaceutical Co., Ltd. An authentic crude drug sample (No. OA20090201) was deposited at the School of Pharmacy, Guiyang Medical University, China.

Cell culture

The human liver cell L02 was maintained in an atmosphere of 5% CO_2 at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100U/mL penicillin and 100 μg/mL streptomycin.

Cytotoxicity assay

L02 cells were cultured in 96-well plates at a density of 3×10^5 cells/mL. The cytotoxicity assay of each isolated compound was performed at concentrations from 0.125 to 1000 μmol/L using the MTT method (Mimaki *et al*., 2001) with OA also as a positive control. All experiments were performed in triplicate.

STATISTICAL ANALYSIS

Data were analyzed using the Dunnett′s test. The data were presented as mean ± SD. $P < 0.05$ was considered as statistically significant.

Isolation

The crude OA drug (915 g) was separated by silica gel column chromatography (CC) gradiently eluted with $CHCl_3$-MeOH (1:0 → 50:1) to give oleanolic acid as the major product (403 g) and six minor fractions (Fr. 1-6). Fr. 1 was subjected to semi-preparative HPLC eluted with 90% *a.q.* MeOH to yield two sub-fractions, 1a and 1b. Purification of the former by silica gel CC eluted with petroleum ether-EtOAc-formic acid (20:1:0.1) afforded **7** (3.5 mg), while that of the latter by silica gel CC eluted with petroleum ether-EtOAc (10:1) yielded **6** (23 mg). Successive separation of Fr. 2 by MCI CC (70% *a.q.* MeOH → 100% *a.q.* MeOH) and semi-preparative HPLC (85% *a.q.* MeOH) gave seven fractions (Fr. 2a - 2g). Purification of Fr. 2a and 2c by Sephadex LH-20 (MeOH)

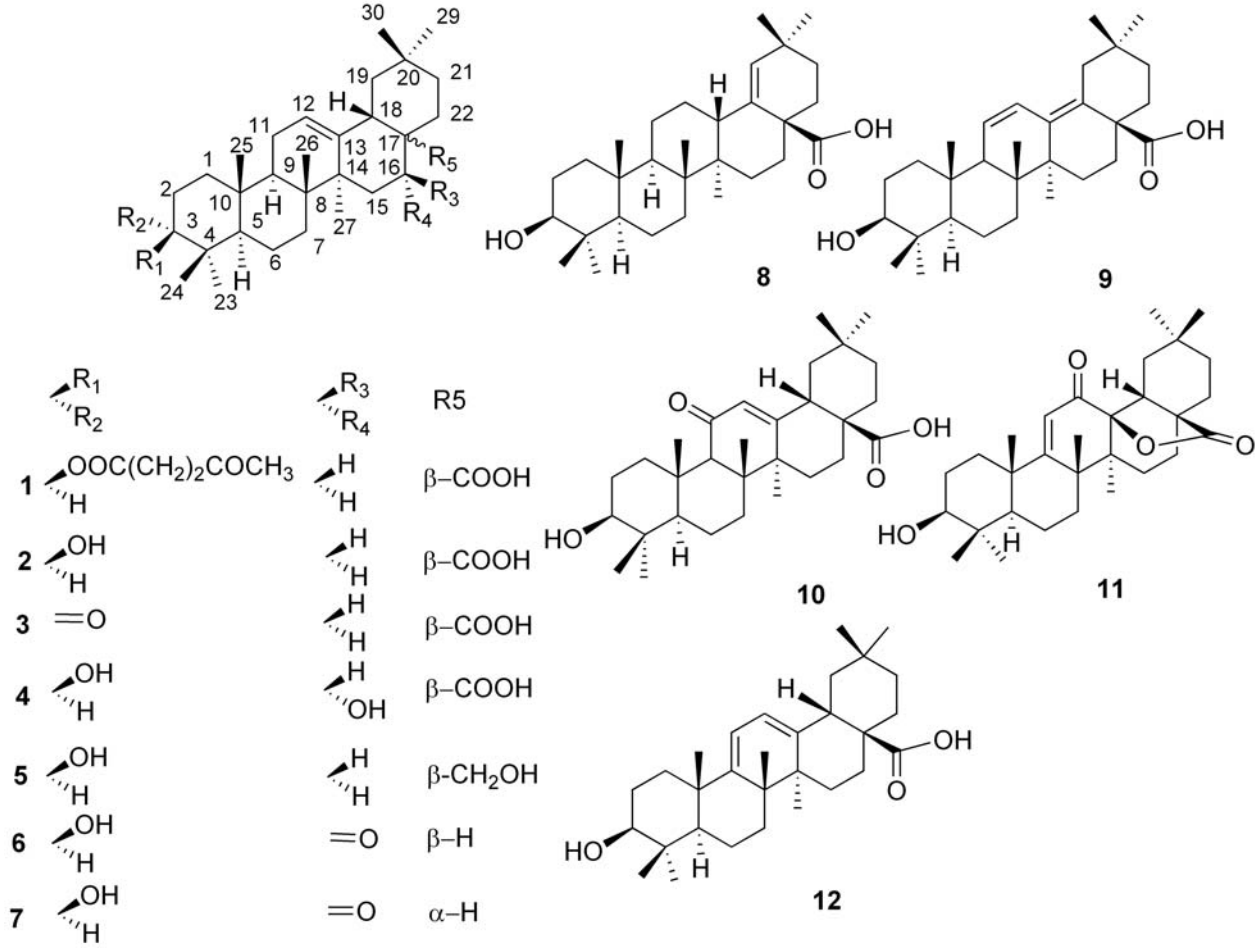

Fig. 1: Structures of compounds **1**-**12** isolated from the Chinese OA crude drug.

and semi-preparative HPLC (85% *a.q.* MeOH) afforded **11** (19 mg) and **9** (51 mg), respectively, while that of Fr. 2b successively by Sephadex LH-20 (MeOH), silica gel CC ($CHCl_3$-MeOH, 300:1), and semi-preparative HPLC (85% *a.q.* MeOH) yielded **12** (45 mg). Purification of Fr. 2d by silica gel CC ($CHCl_3$-MeOH, 300:1) gave **8** (17 mg), while separation of Fr. 2f by silica gel CC ($CHCl_3$) and semi-preparative HPLC (85% *a.q.* MeOH) afforded **5** (7 mg). Fr. 2g was separated by reversed phase C-18 silica gel CC (80% *a.q.* MeOH → 100% *a.q.* MeOH) to give four sub-fractions (Fr. 2g1- 2g4). Fr. 2g4 was further separated by Sephadex LH-20 (MeOH) to afford two fractions, 2g4a and 2g4b, purification of which by silica gel CC (petroleum ether-EtOAc, 5:1) and semi-preparative HPLC (85% *a.q.* MeOH) afforded **1** (7 mg) and **3** (60 mg), respectively. Fr. 6 was separated by semi-preparative HPLC (85% a.q. MeOH) to give two fractions, Fr. 6a and 6b. Purification of the former by silica gel CC ($CHCl_3$-MeOH, 50:1) gave **4** (53 mg), while that of the latter by silica gel CC ($CHCl_3$-MeOH, 60:1) yielded **10** (19 mg)

3-O-(4-Oxo-pentanoyl)-olean-12-en-28-oic acid (1):

White amorphous powder; $[\alpha]_D^{20}$ +36 (*c* 0.22, $CHCl_3$) ESI-MS *m/z*: 577.6 $[M+Na]^+$, 552.8 $[M-H]^-$, 599.1 $[M+HCOO]^-$; HRESIMS *m/z*: 553.3901 (calcd for $C_{35}H_{53}O_5$ 553.3893); IR (KBr) ν_{max}: 2956, 2925, 2857, 2361, 1729, 1690, 1462, 1366 cm^{-1}; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 0.75 (3H, s), 0.85 (3H, s), 0.86 (3H, s), 0.90 (3H, s), 0.92 (3H, s), 0.93 (3H, s), 1.13 (3H, s), 2.19 (3H, s), 2.59 (2H, t, *J* = 5.8 Hz, H_2-3′), 2.77 (2H, t, *J* = 5.8 Hz, H_2-2′), 2.83 (1H, dd, *J* =12.0, 4.2 Hz, H-18), 4.51 (1H, dd, *J* = 8.6, 6.8 Hz, H-3), 5.29 (1H, t, *J* = 3.2 Hz, H-12); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 15.5, 16.7, 17.3, 18.3, 23.1, 23.6, 23.8, 26.1 27.8, 28.2, 28.6, 29.8 (C-5′), 30.0 (C-2′), 30.8, 32.6, 32.7, 33.2, 33.9, 37.1, 37.8 (C-3′), 38.2, 38.3, 39.4, 41.2, 41.8, 46.1, 46.7, 47.7 55.5, 81.4 (C-3), 122.7 (C-12), 143.8 (C-13), 172.6 (C-1′), 183.5 (C-28), 206.9 (C-4′).

Maragenin I (6): White amorphous powder ; IR (KBr) ν_{max} 3458, 2936, 1707, 1460, 1379 cm^{-1}; ESI-MS *m/z*: 427.6 $[M+H]^+$, 409.6 $[M+H-H_2O]^+$, 853.8 $[2M+H]^+$; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 0.78 (3H, s, 0.84 (3H, s), 0.87 (3H, s), 0.92 (3H, s), 0.95 (3H, s), 0.98 (3H, s), 1.01 (3H, s), 2.03-2.16 (3H, m), 2.20-2.30 (1H, m, H-18), 2.52 (1H, d, *J* = 15.5 Hz, H-15a), 3.22 (1H, dd, *J* = 11.0, 4.3 Hz, H-3), 5.46 (1H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 15.4, 15.5, 16.8, 18.5, 23.3, 23.4, 24.8, 25.5, 27.2, 28.1, 30.6, 33.0, 33.2, 36.9, 37.3, 38.2, 38.4, 38.6, 38.8, 42.8, 44.1, 45.5, 49.6, 55.5, 78.9 (C-3), 117.7 (C-12), 142.4 (C-13), 215.7 (C-16).

RESULTS

Compound **1** was assigned the molecular formula of $C_{35}H_{54}O_5$ on the basis of its negative HRESIMS ion peak at *m/z* 553.3901 (calcd for $[M-H]^-$, $C_{35}H_{53}O_5$, 553.3893). Its IR spectrum showed adsorptions for carbonyl (1729 cm^{-1}) and carbon-carbon double bond (1690 cm^{-1}). Comparison of its ^{13}C-NMR data with those of oleanolic acid (**2**) (Ramírez-Espinosa *et al*., 2011) indicated that, apart from signals for an oleanolic acid scaffold, additional signals for a 3- or 4-oxo-pentanoyl group (δ_C 206.9, 172.6, 37.8, 30.0, 29.8) was observed in the ^{13}C-NMR spectrum of **1**. The presence of a singlet acetyl (δ_H 2.19, 3H, s) and two vicinal methylenes [δ_H 2.59 (2H, t, *J* = 5.8 Hz, H_2-3′), 2.77 (2H, t, *J* = 5.8 Hz, H_2-2′] in the ^{1}H-NMR spectrum suggested that this group was a 4-oxo-pentanoyl rather than a 3-oxo-pentanoyl. Observations of the downfield-shifted signals for H-3 (δ_H from 3.20 to 4.51) and C-3 (δ_C from 79.2 to 81.4) indicated that the 4-oxo-pentanoyl group was present as a 4-oxo-pentanoyloxyl at C-3. Compound **1** was therefore possibly a 3-*O*-(4-oxo-pentanoyl) derivative of oleanolic acid. Detailed HMBC correlation analysis further confirmed this conclusion. In particular, HMBC correlations from H_3-5′ to C-4′ and C-3′ , from H_2-2′ to C-4′ and C-1′ and from H_2-3′ to C-4′ and C-1′ supported the presence of the 4-oxo-pentanoyl group, while correlations from H-3 to C-1′ suggested that this group was connected to C-3 through an ester bond (fig. 2). The coupling pattern of H-3 (dd, *J* = 8.6, 6.8 Hz) was very close to that of OA (dd, *J* = 9.2, 6.2 Hz), suggesting that H-3 of **1** was also α-orientated. Compound **1** was thus characterized as 3-*O*-(4-oxo-pentanoyl)-olean- 12-en-28-oic acid. Assignments of the ^{1}H and ^{13}C NMR data were made by a combination of HMQC and HMBC spectroscopic analysis.

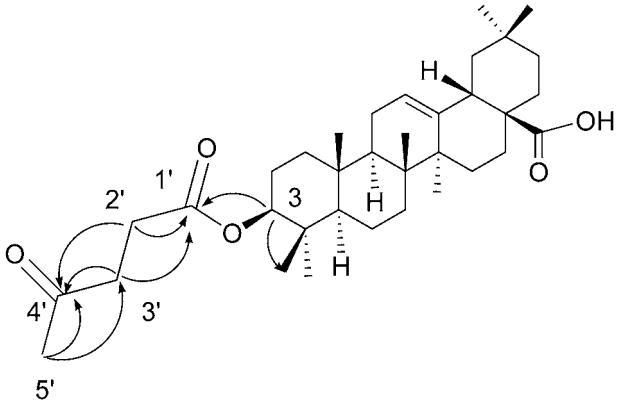

Fig. 2: Key HMBC correlations (H→C) of compound **1**.

The eleven known compounds were identified to be oleanolic acid (**2**) (Ramírez-Espinosa *et al*., 2011), oleanonic acid (**3**) (Lee *et al*., 2005), echinocystic acid (**4**) (Khajuria *et al*., 2007), erythrodiol (**5**) (Lee and Chang, 2000), maragenin I (**6**) (Hylands and Salama, 1979), auriculatone (**7**) (Gao *et al*., 1994), morolic acid (**8**) (Ye *et al*., 1998), 3β-hydroxy-oleana- 11,13 (18)-dien-28-oic acid (**9**) (Ikuta *et al*., 1995), 3β-hydroxy-11- oxoolean-12-

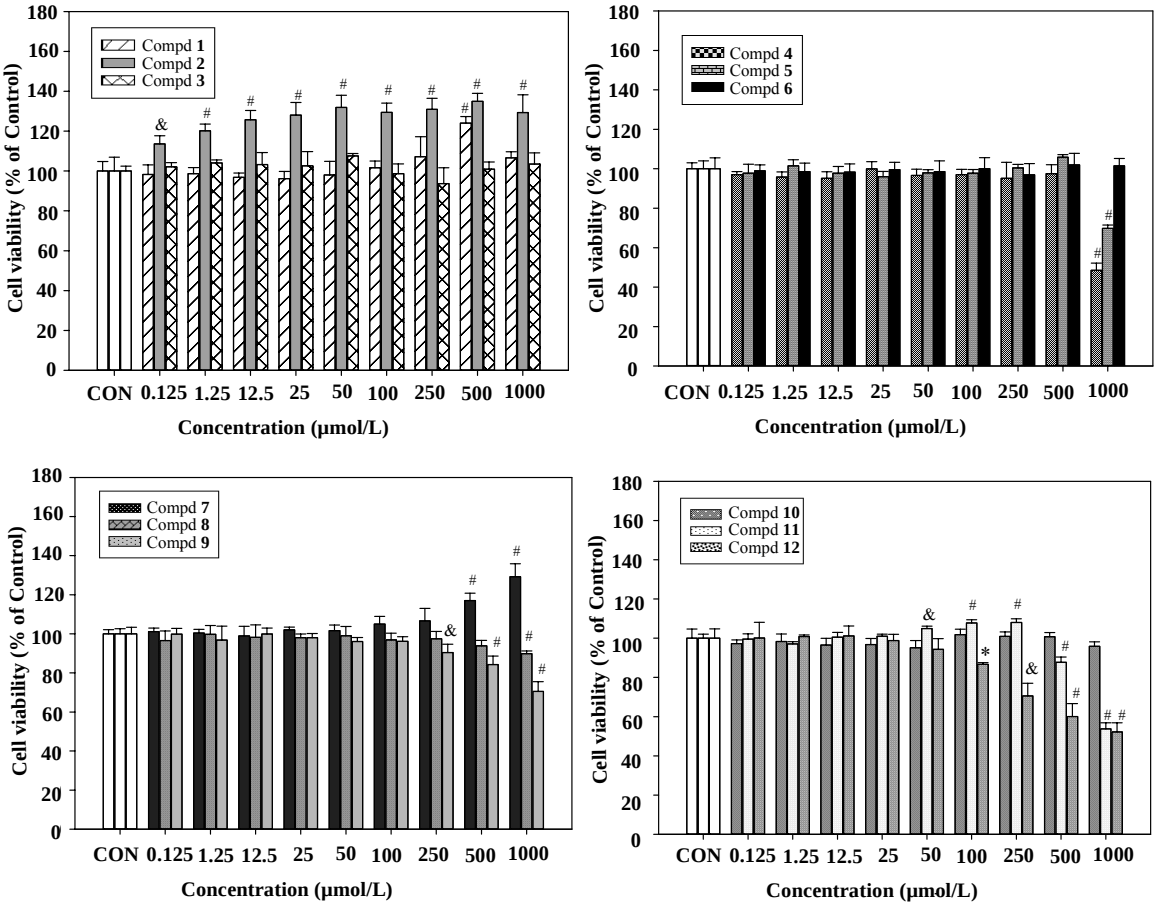

Fig. 3: Cell viability of the L02 cells after treatment of the isolated sapogenins. All experiments were performed in triplicate. Data were presented as means±SD. *P < 0.05 vs. control group; &P < 0.01 vs. control group; #P < 0.001 vs. control group. CON: Control group.

en-28-oic acid (**10**) (Ikuta *et al*., 1995), 3β-hydroxy-12-oxoolean-9(11)-en-28,13β-olide (**11**) (Ding *et al*., 2011), and eucalyptanoic acid (**12**) (Begum *et al*., 2002), by comparing their spectroscopic data with those reported in the literature. ^{13}C-NMR data of maragenin I (**6**), which were not provided in its first report (Hylands and Salama, 1979), have been given in the current investigation.

Each of the 12 compounds was evaluated for its cytotoxicity against the human normal liver cell line L02 (Fig. 3). Despite the fact that these compounds were all non-toxic at concentrations up to 50 μmol/L and compounds **1**, **2**, **3**, **6**, **7**, and **10** were all non-toxic at the tested concentrations (i.e., ≤ 1000 μmol/L), compounds **4**, **5**, **8**, **9**, **11**, and **12** showed cytotoxicity at concentrations of 1000, 1000, 1000, 250, 500, and 100 μmol/L, respectively. The promotion effect on the generation of L02 cells was also observed for OA (**2**) at all tested concentrations and for compound **7** at 500 μmol/L and above.

DISCUSSION

It should be noted that although the crude drug was produced from the acid hydrolysate of *Hemsleya chinensis* Cogn. ex Forb. et Hemsl (Cucurbitaceae), whether these compounds were derived from OA or from other compounds present in the plant was still unknown. Structure-cytotoxicity analysis suggested that oxidation of the hydroxymethine (as in OA) to a carbonyl for C-3 (as in compound **3**) would slightly increase the cytotoxicity of OA, however, acylation of the hydroxymethine (as in compound **1**) had little effect on the cytotoxicity. The latter was in agreement with the conclusion that the presence of an acyl (cinnamoyl as an example) group at C-3 might not always increase antitumor activity (Sun *et al*., 2006). Immigration of the carbon-carbon double bond from a $\Delta^{12(13)}$ (as in OA) to a $\Delta^{18(19)}$ (as in compound **8**) had very little effect on the cytotoxicity, as **8** was only slightly toxic at concentrations of 500-1000 μmol/L.

Introduction of a $\Delta^{9(11)}$ double bond (as in compound **12**) will dramatically increase the cytotoxicity, whereas, oxidation of the CH_2-11 to a carbonyl (as in compound **10**) had almost no effect on the cytotoxicity. Introduction of an α-OH to C-16 (as in compound **4**) slightly increased the cytotoxicity of OA, but oxidation of this carbon to a carbonyl and decarboxylation of C-28 (as in compounds **6** and **7**) would reduce dramatically the cytotoxicity of OA-type triterpenoids. Although some of the structure-hepatotoxicity (Kinjo *et al*., 1999) and structure-cytotoxicity (Sun *et al*., 2006) of OA-type triterpenoids were indicated in the previous reports, detailed structure-cytotoxicity information had not been provided. The structure-cytotoxicity discovered in the present work has added valuable information to the structure-cytotoxicity of OA-type triterpenoids.

CONCLUSION

From the above studies it is concluded that the OA crude drug (and its tablet) contains a number of trace co-occurring substances. The lower cytotoxicities of OA and these compounds against the human normal liver cell line L02 suggested that it was possible that OA tablets of similar origin might not cause obvious cytotoxicity to the normal liver cell. The structure-cytotoxicity relationship revealed in the current investigation may facilitate further structure-cytotoxicity relationship studies of OA-type triterpenoids.

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