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Cytotoxic and antitubercular activity of new lupane-type triterpenoids from the nut of *Anacardium occidentale*

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ABSTRACT

Two new lupane triterpenoids (2, 3) were isolated from the bioactive methanol extract of the fruit (nut) of *Anacardium occidentale*, along with three known compounds. The structures of the new compounds were elucidated based on spectroscopic data analysis. All new triterpenoids were evaluated for their *in vitro* antitubercular activity toward *M. tuberculosis* ATCC 27294. The isolated compounds 2 & 3 were tested *in vitro* for cytotoxic potential against MCF-7 cells (human breast cancer), BGC-823 cells (human gastric carcinoma) and A-549 cells (lung cancer). Compounds 3 exhibited significant cytotoxic activities against all tested tumor cell lines.

KEYWORDS

Anacardium occidentale;
Triterpenoids;
Epi-betulinic acid;
Antitubercular activity.

INTRODUCTION

Anacardium occidentale L. (cashew), family Anacardiaceae, is widely grown in many tropical and subtropical countries. Nuts of *A. occidentale* have been used as a food ingredient, especially in oriental delicacies, and have great economic and medicinal value. β-Sitosterol was identified in the light petroleum extract of the tender leaves of *A. occidentale*, whereas ethyl gallate and methyl gallate were found using two dimensional paper chromatography in methanol extracts[1]. Analysis of the leaves revealed major constituents such as (*E*)-β-cymene, α-copaene and δ-cadinene. The major constituents of the fruit are palmitic acid, oleic acids, furfural, 4-hydroxydodecanoic acid lactone, (*E*)-hexenal, (*Z*)-hex-3-enol and hexadecanol, whereas in the flowers, β-caryophyllene, methyl salycilate and benzyl tiglate were the main constituents[2]. The main aldobiuronic acid present is 6-*O*-(β-D-glucopyranosyl uronic acid)-D-galactose[3]. The major components of the essential oil were cardol, cardanol, and anacardic acid[4]. *A. occidentale* has been used in folk medicine in the treatment of skin and venereal diseases, respiratory problems and nervous disorder[5]. *A. occidentale* leaves, stem and bark extracts have been reported to possess anti-diabetic, anti-bacterial, anti-inflammatory and anti-ulcerogenic properties[6]. Various extracts from other parts of the plants were reported to show breast anti-tumor activity[7].

EXPERIMENTAL

General

Optical rotations were measured in MeOH or H_2O on a Perkin-Elmer241 polarimeter equipped with a sodium lamp (589 nm) and a 1 dmmicrocell. The melting points were determined using a digital

melting point apparatus (model IA 8103, Electro thermal Engineering Ltd, Soutthend-on-Sea Essex, UK). IR spectra (KBr) were recorded on a Perkin-Elmer 1650 FT-IR spectrometer. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Bruker Avance DRX-500 spectrometer (^{1}H at 500 MHz and ^{13}C at 125 MHz), and 2D-NMR experiments were performed using Bruker's standard microprograms (XWIN-NMR version 2.6 software). EIMS were recorded on a Micromass TRIO-2000 GC/MS spectrometer, and HREIMS were recorded on a Finnigan/ Thermo Quest MAT 95. TLC was carried out on precoated silica gel 60 F254 (Merck), and spots were visualized by heating after spraying with 50% H_2SO_4. Column chromatography was carried out on silica gel 60 (63–200 μm, Merck).

Plant material

The nuts of *A. occidentale* L. were collected from a local market in Saudi Arabia (May, 2013), authenticated by Prof. Dr. F. Gamal, Head of Botany Department, Faculty of Science, Zagazig University, Zagazig, Egypt. A herbarium of the plant specimen (voucher number no. YOGA1 / No. BSI/ WC/ Tech/ 2008/69) was submitted at the same institute in Egypt.

Plant extract

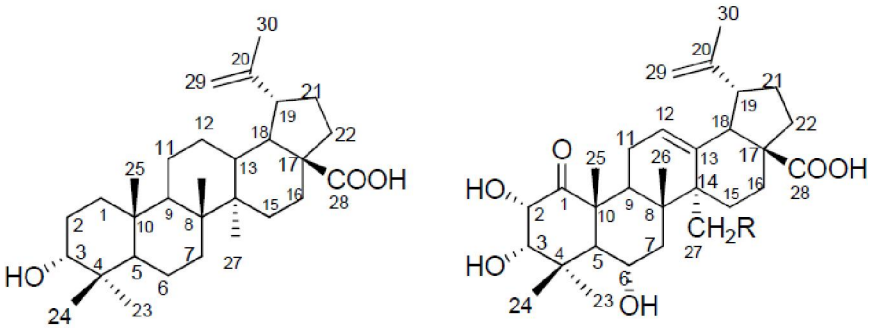
The dried ground seeds of A. occidentale (1.5 kg) were exhaustively extracted with petroleum ether, EtOAc and MeOH successively. The extracts were concentrated In vacuo to give yellow solid (12.8 g), yellow brownish solid (21.2 g) and brown solid (15.4 g), respectively. The MeOH extract (12 g) was further partitioned using H_2O, MeOH and $CHCl_3$ (1:1:1) to give the $CHCl_3$ extract. The $CHCl_3$ extract (5 g) was chromatographed on a silica gel column eluting with increasing gradient of *n*-hexane–EtOAc to give seventy 100 mL fractions. Similar fractions as determined by TLC were pooled together giving six combined fractions. Fraction 3 eluted with *n*-hexane/EtOAc (2:1) afforded 1 (22 mg) purified by recrystallization in petroleum ether. Fraction 7 eluted with 100% EtOAc was subjected to further purification by recrystallization in petroleum ether to give 2 (14 mg, t_R = 2.4 min) and 3 (19 mg, t_R =3.8 min). See Figure 1.

1-oxo-2α, 3α, 6α-trihydroxylup-12-en-28-oic acid (2)

- m.p. 218–220°C
- $[\alpha]_D$:+37.2 (*c* 1.00, $CHCl_3$)
- R*f*: 0.39 ($CHCl_3$–MeOH, 1:1)
- IR (KBr) νcm^{-1}: 3541, 2955, 1710, 1635, 1430, 1381, 1294, 1161, 1081, 1036, 941
- ^{1}H NMR (500 MHz, $CDCl_3$): TABLE 1
- ^{13}C NMR (125 MHz, $CDCl_3$): TABLE 1
- MS (EI, 70 eV): *m/z* (%) = 466 $[M - 2OH]^+$ (23), 455$[M - COOH]^+$ (11), 419 $[M - COOH_3 + 2OH]^+$ (17), 285 (43), 270 (22), 264 (17),204 (100), 136 (3).
- HR-EIMS: *m/z* $[M]^+$ calcd for $C_{30}H_{44}O_6$: 500.6662; found: 500.6153.
- Anal. Calcd for $C_{30}H_{44}O_6$: C, 71.95; H, 8.85; O, 19.17. Found C, 71.92; H 8.81; O, 19.14

1-oxo-2α, 3α, 6α, 27-tetrahydroxylup-12-en-28-oic acid (3)

- m.p. 214–216°C



1

2, R=H
3, R=OH

Figure 1 : Structures of isolated compounds

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TABLE 1 : NMR spectroscopic data of isolated compounds in $CDCl_3$

Positions	2		3	
	^1H	^{13}C	^1H	^{13}C
1	-	211.2	-	214.6
2	4.3 dd(12.1,10.6)	79.1	3.5 dd(11.2,6.8)	78.2
3	3.58d(2.5)	91.6	3.44d(3.12)	91.6
4	-	35.9	-	36.2
5	1.5m	51.3	1.52m	51.8
6	3.27 br s	68.4	3.19 br s	68.8
7	1.64m,1.32m	42.8	1.66m,1.39m	44.2
8	-	29.1	-	48.7
9	1.61m	37.4	1.54m	37.4
10	-	41.9	-	40.7
11	2.11m,1.72m	24.3	2.21m,1.72m	24.9
12	5.82(dd2.1,8.2)	123.6	5.76(dd1.7,8.5)	124.1
13	-	142.3	-	140.1
14	-	27.6	-	57.1
15	1.28m, 1.13m	24.2	1.28m, 1.13m	46.1
16	1.61m, 1.36m	23.6	1.61m, 1.36m	44.2
17	-	46.7	-	46.7
18	2.51(dd3.2,8.1)	41.2	2.51(dd3.2,8.1)	42.1
19	2.23d	49.3	2.23d	48.3
20	-	141.2	-	142.3
21	1.61m, 1.42m	27.3	1.61m, 1.42m	27.3
22	1.83m, 1.53m	31.9	1.83m, 1.53m	30.5
23	1.02s	23.6	0.99s	23.2
24	1.02s	23.6	0.99s	23.2
25	1.04s	16.5	1.02s	16.2
26	1.66s	17.5	1.61s	17.5
27	1.31s	25.7	2.39m,3.13m	65.4
28	-	180.2	-	182.5
29	5.11 br s,4.91 br s	110.1	5.12 br s,4.63 br s	111.4
30	1.82s	23.8	1.77s	24.1
2-OH	2.85	-	2.81	-
3-OH	3.51	-	3.58	-
6-OH	3.51	-	3.58	-

- $[\alpha]_D$:+39.1 (*c* 1.00, $CHCl_3$)
- R*f*: 0.32 ($CHCl_3$–MeOH, 1:1)
- IR (KBr) νcm^{-1}: 3491, 2968, 1720, 1648, 1632, 1561, 1458, 1373, 1290, 1247, 1229, 1172, 1042, 971
- ^1H NMR (500 MHz, $CDCl_3$): TABLE 1
- ^{13}C NMR (125 MHz, $CDCl_3$): TABLE 1
- MS (EI, 70 eV): *m/z* (%) = 482 $[M - 2OH]^+$ (11),471$[M - COOH]^+$ (24), 435 $[M - COOH_3 + 2OH]^+$ (17), 288 (54), 270 (24), 264 (13),204 (100),179(11), 149 (3).
- HR-EIMS: *m/z* $[M]^+$ calcd for $C_{30}H_{44}O_7$: 516.6659; found: 516.6171.
- Anal. Calcd for $C_{30}H_{44}O_7$: C, 69.72; H, 8.58; O, 21.67. Found C, 69.71; H, 8.55; O, 21.64

Antitubercular activity

Antitubercular activities of compounds 2 and 3

were assessed against *M. tuberculosis* H_{37}Rv ATCC 27294 using the micro plate Alamar blue assay (MABA)[8]. The minimal inhibitory concentration (MIC) of the compound was defined as the concentration necessary to inhibit 90% of the mycobacterial growth in sterile 96-well plates (Falcon 3072; Becton Dickinson, Lincoln Park, NJ). Fluorescence was measured in a Spectrafluor Plus micro-fluorimeter (Tecan) in bottom reading mode, with excitation at 520 nm and emission at 590 nm. A sample with a MIC value <125 μg/mL was defined as active against *M. tuberculosis*[9]. As a standard control, the MIC value of isoniazid was determined on each microplate. The acceptable MIC of isoniazid ranged from 0.015 to 0.05 μg/mL[8].

In vitro cytotoxicity assay

The isolated compounds 2 & 3 were subjected to cytotoxic evaluation against A-549 cells (lung cancer), BGC-823 cells (human gastric carcinoma) and MCF-7 cells (human breast cancer) which, were obtained from Thessaloniki, Institute of Medical Science, Greece by employing the revised MTT method as described in the literature[10]. Doxorubicin was used as the positive control. All tumor cell lines were cultured on RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U mL^{-1} penicillin and 100 μg/mL streptomycin in 25 cm^3 culture flasks at 37 °C in humidified atmosphere with 50% CO_2. For the cytotoxicity tests, cells in exponential growth stage were harvested from culture by trypsin digestion and centrifuging for 3 min, and then resuspended in fresh medium at a cell density of 5×10^4 cells per mL. The cell suspension was dispensed into a 96-well microplate at 100 μL per well, and incubated in humidified atmosphere with 50% CO_2 at 37 °C for 24 h, and then treated with the compounds at various concentrations (10, 50, 100 μM). After 48 h of treatment, 50 μL of 1 mg/mL MTT solution was added to each well, and further incubated for 4 h. The cells in each well were then solubilized with DMSO (100 μL for each well) and the optical density (OD) was recorded at 570 nm. All drug doses were tested in triplicate and the IC_{50} values were derived from the mean OD values of the triplicate tests *versus* drug concentration curves. The 50% inhibition concentration (IC_{50} value) was determined by curve fitting and was used as criteria to judge the cytotoxicity (active: $IC_{50} \geq 20$ μM; moderately active: 20 μM < $IC_{50} \leq 80$ μM; not active: IC_{50} > 80 μM).

RESULTS AND DISCUSSIONS

Chemistry

From the methanol, extract compound 2 was obtained as colorless needles, mp 218–220 °C. Its molecular formula was determined to be $C_{30}H_{44}O_6$ by positive mode HR-EIMS {$[M]^+$ *m/z* 500.6153; calcd 500.6662). The ion at m/z 270 corresponding to the molecular formula $C_{15}H_{25}O_4^+$ indicated three hydroxyl groups were seemed to be located in the two rings A and B. The IR spectrum displayed the absorptions for OH (3541 cm^{-1}), C=O (1710 cm^{-1}) and C=C (1635 cm^{-1}) groups. The ^{13}C NMR spectrum (TABLE 1) of 2 revealed thirteen carbon signals, which were deduced as six methyl, seven methylene, five methine, eight quaternary carbons, three secondary alcohols and one carboxylic acid. The ^{1}H NMR spectrum of 2 showed an olefinic proton signal (δ_H 5.82, dd), three secondary alcohol groups at δ_H 2.85 (br s, 2-OH) and δ_H 3.51 (br s, 3, 6-OH), in addition to six tertiary methyl groups [δ_c 1.02, 1.02, 1.04, 1.31, 1.66, 1.82 (each 3H, s)] (TABLE 1). From the NMR spectra, an isopropenyl group [δ_H 1.82 (3H, s), 5.11 (1H, br s), 4.91 (1H, br s); δ_c 23.8, 110.1, 141.2] and one carbonyl group [δ_c 180.2] were observed, suggested 2 to be a pentacyclic triterpene of the lupane family[11, 12]. Assignments of the ^{1}H and ^{13}C signals by 2D NMR spectra revealed that 2 was an analogue of lup-12-en-28-oic acid. The presence of the double bond at C-12 was confirmed by the chemical shifts of C-12 (δ_c 123.6) and C-13 (δ_c 142.3), characteristic of a "^{12} skeleton[13]. The remaining signal was characteristic for the oxygenated carbons, C-1 (δ_c 211.2). The ^{13}C NMR analyses showed, no resonance for any oxygenated methylene and displayed instead to carbonyl signals at δ_c 180.2 indicated, a carbon atom bearing two oxygen, were assigned to a carboxyl groups at the C-28 positions. Based on the HMBC spectrum (Fig-

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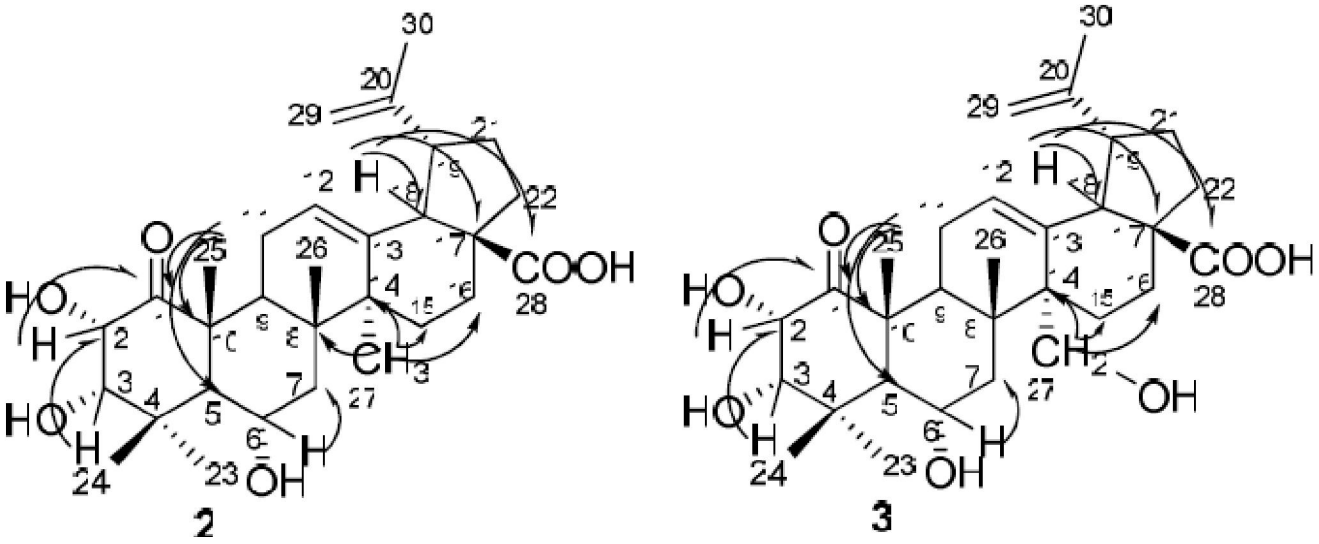

Figure 2 : HMBC correlation of compounds 2 and 3

ure 2), in which the H-18 [δ_H, 2.51(dd, J = 3.28.1 Hz)] exhibited a correlation with the carboxyl group (δ_c 180.2)[14]. ^{13}C NMR showed, the one downfield quaternary carbon signal at δ_c, 211.2 (C-1) and one downfield methine carbon signal 79.1 (C-2) showed the presence of ketonic and one hydroxyl functionality in the molecule[15]. The location of the ketone group at C-1 was confirmed by the HMBC correlations of H_3-25 (δ_H 1.04) with C-1 (δ_c 211.2) and of H-2 (δ_H 4.3) with C-1 (δ_c 211.2). Also, the HMBC correlations of H-3 (δ_H 3.58) with C-2 (δ_c 79.1) and the correlations of H-2 (δ_H 4.3) and H-3 (δ_H 3.58) with C-24 (δ 23.6) indicated that the two hydroxyl groups should be located at C-2 and C-3[16]. In addition, the primary alcohol at C-6 confirmed by HMBC correlations with C-8 (δ_c 29.1), C-13 (δ_c 142.3), C-14 (δ_c 47.6), and C-15 (δ_c 24.2). While the C-27 methyl protons at δ_c 1.02 showed, correlations with C-14 (δc 27.6), C-15 (δc 24.2), and C-16 (δc 23.6) suggested, methyl group was attached to C-14 (Figur 2). The ^{13}C NM R signals at C-2 (δc 79.1), C-3 (δc 91.6) and C-6 (δc 68.4) and its corresponding methine proton signals at H-2 (δ_H 4.3 dd, J = 12.1, 10.6 Hz), H-3 (δ_H 3.58 d, J = 2.5 Hz) and H-6 (δ_H 3.27 br s), suggested the hydroxyl groups at C-2, C-3 and C-6 are α-substituted. In addition, the splitting nature with high J values indicated, the three hydroxyl groups at α position[17, 18]. Based on the data above, compound 2 was designated as 1-oxo-2α, 3α, 6α-trihydroxylup-12-en-28-oic acid.

Compound 3 obtained as colorless needles, mp 214–216°C. Its molecular formula was determined to be $C_{30}H_{44}O_7$ by positive mode HR-EIMS { $[M]^+$*m/z* 516.6171), indicating16 mass units larger than that of 2, suggesting replacement of the hydrogen of 2 by an OH in 3 and was confirmed by ^{13}C NMR analyses. The methine proton from ^{13}C NMR spectra resonance at H-2 (δ_H 3.5 dd), H-3(δ_H 3.44 d), H-6 (δ_H 3.19 br s) and 2H-27 (δ_H2.39m,3.13m) suggesting that the hydroxyl groups at C-2, C-3, C-6 and C-27 are α-substituted[17,18]. The ^{1}H and ^{13}C NMR data were closely related to those of 2. The position of the C-27 hydroxyl group was established from the HMBC data, in which the H-27b proton at δ_H 3.13 showed long-range correlations with C-8 (δ_c 48.7), C-14 (δ_c 57.1), C-15 (δ_c 46.1), and C-16 (δ_c 44.2). The presence of the double bond at C-12 was confirmed by the chemical shifts of C-12 (δ_c 123.9) and C-13 (δ_c 144.9). The methine proton from ^{13}C NMR spectra resonance at H-2 (δ_H 3.58, dd, J = 11.2, 6.8 Hz), H-3 (δ_H 3.44, d, J = 3.12 Hz) and H-6 (δ_H 3.19 br s), suggesting that the hydroxyl groups at C-2, C-3, C-6 and C-27 are α-substituted[17, 18]. In addition, the MS fragmentation at *m/z* 270 indicated that the three hydroxyl groups were seemed to be located in the two rings A and B (Figure 3). Therefore, the structure of 3 was elucidated as 1-oxo-2α, 3α, 6α, 27-tetrahydroxylup-12-en-28-oic acid.

Besides compounds 2 and 3, three known compounds were isolated from *A. occidentale* namely,

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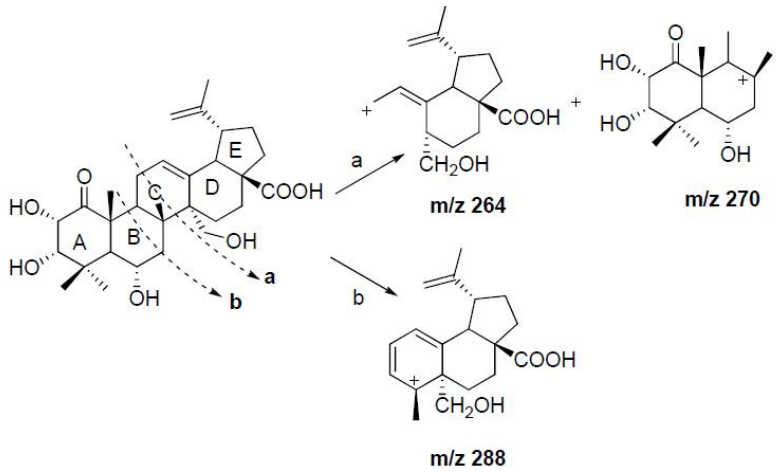

Figure 3 : Mass fragmentation patterns of 3

TABLE 2 : Vitro antitubercular values of Compounds 2 & 3 determined by MABA

Compound	Molecular formula	MIC (µg/mL)
2	$C_{30}H_{44}O_6$	31.25
3	$C_{30}H_{44}O_7$	62.50
Isoniazid		0.03

TABLE 3 : In vitro cytotoxicity of compounds 2&3 against three human tumor cell lines (IC_{50}, µM)

Compound	Molecular formula	IC_{50} MCF-7	BGC-823	A-549
2	$C_{30}H_{44}O_6$	33.1	30.2	29.8
3	$C_{30}H_{44}O_7$	13.9	11.2	7.5
Doxorubicin		18.2	21	22

Doxorubicin activities are expressed as IC50 values and those of compounds 2&3 are expressed as IC_{50} values in µM. (-) IC_{50} > 100 µM.

3-*epi*-betulinic acid (1)[19], *α*-amyrin[20] and *β*-sitosterol[21]. The structures of the known compounds were determined by a combination of spectroscopic analysis and comparison with previously reported data.

Antitubercular activity

Based on the previous literatures, bioactive compounds, which have been isolated from plant extracts, were an attractive source of new drugs. On this basis, the antitubercular activities of compounds 2 and3 were assessed against *M. tuberculosis* ATCC 27294[8], using the micro plate Alamar Blue assay (MABA). This methodology is non-toxic. These results showed that MICs 31.25 µg D mL for compound 2 and 62.50µgD mL for compound 3 (TABLE 2).

Cytotoxic activity

Compounds 2 & 3 were evaluated for their *in vitro* cytotoxic potential against three tumor cell lines and the results were summarized in TABLE 3. Triterpenoids 3 exhibited significant cytotoxicity against MCF-7 cells (human breast cancer), BGC-823 cells (human gastric carcinoma) and A-549 cells (lung cancer), with IC_{50} values of 13.9, 11.2 and 7.5, respectively. Triterpenoids 2 showed moderate cytotoxic activities against all assayed three tumor cell lines (20 µM < IC50 ≤ 50 µM). The Triterpenoids 2 & 3 isolated from *A. occidentale* shared the same basic skeletal structure with a wide variety of side chains. Due to their intrinsic structural variety and impressive biological activities, we suggest that the increasing of hydroxy group might strengthen the cytotoxic action.

CONCLUSION

In conclusion, two new lupane-type triterpenoids

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are isolated from the nut of *A. occidentale* (Cashew). Based on spectroscopic techniques and chemical evidences, structures were elucidated as 1-oxo-2α, 3α, 6α-trihydroxylup-12-en-28-oic acid (**2**) and 1-oxo-2α, 3α, 6α, 27-tetra hydroxy lup-12-en-28-oic acid (**3**). The present study indicated that two compounds **2** and **3** have antitubercular activity toward *M. tuberculosis* ATCC 27294. Compounds **2** & **3** exhibited significant cytotoxicity against MCF-7 cells (human breast cancer), BGC-823 cells (human gastric carcinoma) and A-549 cells (lung cancer).

Conflict of interest

The authors confirm that this article content has no conflict of interest.

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