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Chemical Profile and Cytotoxicity Activity of Stem-bark of *Anacardium occidentale*

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Abstract – Column chromatographic fractionation of the methanol and ethyl acetate extracts of the stem-bark of *Anacardium occidentale* led to the isolation of five compounds (**1-5**). Their structures were determined by spectroscopic means by comparing spectral data to be β-sitosterol (**1**), 2,4-dihydroxy acetophenone (**2**), 1-monolinolein (**3**), ethyl oleate (**4**) and β-sitosterol-3-O-β-D-glucopyranoside (**5**). These compounds were evaluated for cytotoxicity against human cancer cell lines: A549, SCOV3 and rat normal cell line NRK49f. Compounds 2-5 were for the first time isolated from *A. occidentale*.
Keywords – Anacardium occidentale, Anacardiaceae, β-sitosterol, MTT assay, cytotoxicity

Introduction

The genus *Anacardium*, family *Anacardiaceae* comprises about 15 species mainly tropical trees and shrubs which are distributed in Africa, Brazil and India.[1] *Anacardium occidentale* plant is one of the main sources of traditional medicine extensively used in treating various diseases contributing to health benefits all over the world.[2,3] In Africa and India, the barks of *A. occidentale* are used mainly as a medication for ulcer, skin injury and lower extremity pains. In other areas, the juice from the plant apple is used to make a drink against kidney diseases, cholera, syphilis and as astringent and diuretic.[2] Native people in the Republic of Guatemala in Central America use the root maceration for stomach ache, diarrhoea, and as laxative. In Benin Republic, the oil from the plant nut is used as antihypertensive, purgative, antifungal and for healing cracking heels.[2] The plant liquid nuts are widely used in Africa as drinks for rheumatism, heart palpitation and mental derangement. The syrup obtained from the plant is said to be a good medicine for cold and coughs.[2] The kernels from the plant have a soothing and laxative effect. It is used in some part of Africa as emollient, demulcent and for the treatment of diarrhoea.[2] In Eastern Cuba, the barks of *A. occidentale* are pounded with epicarp of *Punica granatum* as a medicine for stomach ulcer. In another herbal preparation, the bark of the plant is mixed with aerial part of *Senna alata* and stem of *Thiounea elliptica* taken as a decoction for diabetes.[3] The plant is used in folk medicine as a raw materials for medicinal preparations, for example in traditional African medicine it is used for the treatment of inflammation and diarhoea.[4] Previous biological activity investigations have shown that *A. occidentale* possessed antibacterial,[5,6] antioxidant,[7,8] antifungal,[9,10] antidiabetic,[11] anticancer[12,13] activities. Phytochemical analysis revealed that *A. occidentale* contain various secondary metabolites in its leaf, shoot, fruit and stem-bark. Phenols and tannins,[14,15] flavonoids,[16] sterols,[17,18] carotenoids,[19] terpenes[20] were reported from *A. occidentale*. In our previous paper, we reported new steroid ester and cerebrosides as well as other two known compounds from *A. occidentale*.[21] Our continuing investigations on the constituents of methanol and ethyl acetate extracts of this plant led to isolation of five known compounds (**1-5**) of which compounds **2-5** were reported for the first time from *A. occidentale*.

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Experimental

General Experimental Procedure – The IR spectra were recorded on a Nicolet-740 FT-IR Spectrometer (Thermo Scientific, Waltham, MA, USA). The NMR spectra were recorded with Bruker AVANCE II (600 MHz), Bruker AVANCE (500 MHz), Varian Inova (400 MHz) and Bruker AVANCE (300 MHz) (Bruker, Billerica, MA, USA) for ^{1}H-NMR, and 150 MHz,125 MHz, 100 MHz, 75 MHz for ^{13}C-NMR in $CDCl_3$, C_5D_5N and $(CD_3)_2CO$ with tetramethylsilane (TMS) as an internal standard to 0 ppm. Coupling constants are given in Hz. The ESI-MS data were recorded on an Agilent 1100 MSD (Agilent Technologies, Santa Clara, CA, USA) with ESI SL Trap. The HR-ESI-MS data were acquired on an Agilent 6510 Q-T.O.F. (Agilent Technologies, Santa Clara, CA, USA and ESI probe. Thin-layer chromatography (TLC) was performed on precoated silica gel GF_{254} plates (Merck & Co., Readington, NJ, USA). The TLC plates were visualized under UV light or by spraying with 5% H_2SO_4 in methanol and heating at 70ºC.

Plant Materials – The stem-barks of *A. occidentale* were collected at Olorunsogo Area in Ilorin, Kwara State. They were identified and authenticated by Mr. Edward Bolu Ajayi at the Herbarium of the Department of Plant Science, University of llorin, Nigeria. Voucher Specimens (UIH 001/970) were deposited at the Department of Plant Science, University of Ilorin, Nigeria.

Extraction and isolation – The powdered, dry stem-bark of *A. occidentale* (1.8 kg) were extracted and fractionated as described before.[21] The ethyl acetate extract (204 g) was subjected to VLC on silica gel (230-400 mesh) eluting with a mixture of acetone hexane to obtain six fractions (EAF_1-EAF_6) base on TLC analysis. Fraction EAF_2 (16.3 g) was further fractionated on a VLC with acetone-hexane mixture to six subfractions (EAF_{2a}-EAF_{2f}). Fraction EAF_{2c} (3.8 g) deposited an impure solid at the base of the test-tube. The impure solid was recrystallized in MeOH at 65ºC to obtain **1** (35 mg). Column chromatographic purification of EAF_{2d} (5.1 g) with acetone-hexane mixture afforded **2** (42 mg) and **3** (8 mg). The ethanol extract (196 g) was subjected to VLC on silica gel (230-400 mesh) and eluted with a mixture of $CHCl_3$-acetone-MeOH in order of increasing polarity to obtain eight fractions (ETF_1-ETF_8). Column chromatographic purification of fraction ETF_1 (4.2 g) on a silica gel with DCM/hexane mixture yielded **4** (25 mg). The more polar fraction ETF_5 (43.2 g) afforded **5** (15 mg) on purification on column chromatography with MeOH-$CHCl_3$ mixture.

Cytotoxicity Assay (MTT Assay) – Human tumour cell lines, A549 (lung adenocarcinoma), SKOV3 (ovarian carcinoma) and normal cell line NRK-49F (normal rat kidney fibroblast) are seeded onto 96-well micro titer plates at different concentrations and then incubated at 36.5ºC in humidified CO_2 5% incubator for 24 hours. The old medium was removed and fresh medium added. 2 µl containing various concentrations of compounds **1**, **2**, **3** and **5** was added whereas positive and negative control has standard drug (doxorubicin) and no standard drug respectively and the plates were incubated for the next 24 hours. After incubation, medium was removed from the wells and 100 µl of fresh medium + 5 µl MTT was added. Plates were incubated for 30-60 minutes, medium/MTT was removed and insoluble product was dissolved in 50 µl DMSO. Finally, the absorbance was measured at 540 nm. The 50% inhibitory concentration (IC_{50}) value of the compounds was calculated.[30]

β-Sitosterol (1) – white amorphous powder; IR ν_{max} cm^{-1}: 3387, 2924, 2853, 1649, 1457, 1349, 1032; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ_H 0.70 (3H, s, H-18), 0.85 (3H, t, J = 6.5 Hz, H- 29), 0.92 (3H, d, J = 6.5 Hz, H-26), 0.95 (3H, d, J = 6.5 Hz, H-21), 1.03 (3H, s, H-19), 3.54 (1H, m, H-3) 5.35 (1H, bd, J = 5.0 Hz, H-6); ^{13}C-NMR ($CDCl_3$, 125 MHz): δ_C 11.8 (C-29), 12.0 (C-18), 18.8 (C-19), 19.0 (C-21), 19.4 (C-26), 19.8 (C-27), 21.0 (C-11), 23.0 (C-28), 24.3 (C-15), 26.0 (C-23), 28.2 (C-16), 29.7 (C-25), 31.6 (C-8), 31.9 (C-2), 32.2 (C-7), 33.9 (C-22), 36.1 (C-20), 36.5 (C-10), 37.2 (C-1), 40.0 (C-12), 42.3 (C-13), 45.8 (C-4), 45.8 (C-24), 50.1 (C-9), 56.0 (C-17), 56.7 (C-14), 71.7 (C-3), 121.7 (C-6), 140.7 (C-5); GC-MS: *m/z* 414.0 $[M]^+$.

2,4-Dihydroxyacetophenone (2) – off-white powder; IR ν_{max} cm^{-1}: 3418, 2927, 2857, 1670, 1606, 1453, 1117, 770; ^{1}H-NMR (acetone-d_6, 300 MHz): δ_H 2.60 (3H, s, H-8), 6.88 (1H, d, J = 8.1 Hz, H-6), 7.45 (1H, d, J = 8.2 Hz, H-5), 7.52 (1H, s, H-3);); ^{13}C-NMR (acetone-d_6, 75 MHz): δ_C 206.5 (C-7), 168.9 (C-2), 167.7 (C-4) 134.6 (C-6), 123.6 (C-1), 117.5 (C-5), 115.7 (C-3), 23.4 (C-8); ESI-MS: *m/z* 153 $[M+H]^+$.

1-Monolinolein (3) – colourless oil; IR ν_{max} cm^{-1}: 3397, 2922, 2854, 1733, 1646, 1465, 1419, 1384, 1053, 721; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ_H 0.87 (3H, t, H-18), 1.25 (saturated CH_2: H-4, H-5, H-6, H-7, H-15, H-16, H-17), 1.6 (2H, m, H-3), 2.03 (6H, m, allylic CH_2: H-8, H-11, H-14), 2.33 (2H, t, J = 7.2 Hz, H-2), 3.59-4.15 (5H, m, H-1', H-2', H-3'), 5.34 (4H, m, olefinic CH: H-9, H-10, H-12, H-13); ^{13}C-NMR ($CDCl_3$, 125 MHz): δ_C 14.0 (CH_3, C-18), 29.0-29.7 (CH_2, C-4, C-5, C-6, C-7, C-15, C-16, C-17) 22.6 – 27.1 (CH_2, allylic, C-8 C-11, C-14) 63.2 (C-1'), 65.0 (C-3'), 70.1 (C-2'), 129.6-129.9 (olefinic carbons: C-

9, C-10, C-12, C-13), 174.2 (carbonyl carbon (C-1); ESI-MS: *m/z* 377 $[M+Na]^+$.

Ethyl oleate (4) – colourless oil; IR ν_{max} cm^{-1}: 2926, 2855, 1694, 1618, 1466, 1325, 1025; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ_H 0.88 (3H, t, *J* = 6.5 Hz, H-18) 1.24 (3H, t, *J* =7.0 Hz, H-2') 1.25 – 1.29 (20H, m, Saturated CH_2: H-4, H-5, H-6, H-7, H-12, H-13, H-14, H-15, H-16, H-17), 1.61 (2H, m, H-3), 2.01 (2H, q, *J* = 6.5 Hz, H-8, H-11), 2.27 (2H, t, *J* =7.5Hz, H-2), 4.13 (2H, q, *J* =7.0 Hz, H-1'), 5.34 (2H, m, H-9, H-10); ^{13}C-NMR ($CDCl_3$, 125 MHz): δ_C 14.1 (C-18), 14.3 (C-2'), 22.7 (C-17), 25.0 (C-3), 27.2 (C-8), 27.2 (C-11) 29.0 – 29.8 (C-4, C-5, C-6, C-7, C-12, C-13, C-14, C-15), 31.9 (C--16), 34.4 (C-2), 60.1 (C-1'), 129.7 (C-9), 130.0 (C-10), 173.9 (C-1); GC-MS: *m/z* 310.0 $[M]^+$.

β-Sitosterol-3-O-β-D-glucopyranoside (5) – amorphous powder; IR ν_{max} cm^{-1}: 3419, 2925, 2855, 1633, 1458, 1385, 1023, 763; ^{1}H-NMR ($CDCl_3$ and CD_3OD, 300 MHz): δ_H : 0.69 (3H, s, H-18), 0.88 (3H, d, *J* = 7.0 Hz, H-27), 0.89 (3H, t, *J* = 7.4 Hz, H-29), 0.92 (3H, d, *J* = 7.3 Hz, H-26), 0.95 (3H, s, H-19) 1.02 (3H, d, *J* = 6.5 Hz, H-21), 3.87 (2H, m, H-3, and H-5'), 4.07 (1H, brt, *J*=8.1Hz, H- 2'), 4.31 (2H, m, H-3' and H-4'), 4.43 (1H, dd, *J* =11.7, 5.2 Hz, H-6') 4.59 (1H, dd, *J* = 11.7, 5.2 Hz, H-6'), 5.06 (1H, d, *J* = 7.7 Hz, H-1') 5.37 (1H, m, H-6); ^{13}C-NMR ($CDCl_3$ and CD_3OD, 300 75 MHz): δ_C 37.6 (C-1), 30.0 (C-2), 79.4 (C-3), 38.9 (C-4), 140.7 (C-5) 122.4 (C-6), 32.2 (C-7), 32.2 (C-8), 50.6 (C-9), 37.0 (C-10), 21.4 (C-11), 40.0 (C-12), 42.7 (C-13), 56.4 (C-14), 24.6 (C-15), 28.6 (C-16), 57.1 (C-17), 12.1 (C-18), 19.2 (C-19), 36.5 (C-20), 19.0 (C-21), 34.3 (C-22), 26.4 (C-23), 46.2 (C-24), 29.5 (C-25) 19.5 (C-26), 20.0 (C-27), 23.4 (C-28), 12.1 ((C-29), 101.5, (C-1'), 76.3 (C-2'), 76.9 (C-3'), 70.5 (C-4'), 79.4 (C-5'), 62.1 (C-6'). HRESI-MS: *m/z* 599.429 $[M+Na]^+$ (calcd for $C_{35}H_{60}O_6Na$; 599.4282).

Results and Discussion

The column chromatographic separation of the methanol and ethyl acetate extract of stem-bark of *A. occidentale* led to the isolation of five compounds **1-5** (Fig. 1). The structures of **1-5** were identified to be β-sitosterol,[22] 2,4-dihydroxyacetophenone,[23] 1-monolinolein,[24] ethyl oleate[26,27] and β-sitosterol-3-O-β-D-glucopyranoside.[28] Compounds **2-5** were isolated for the first time from *A. occidentale*.

Compound **1** was obtained as white powder. The IR spectrum showed absorption band for the presence of OH functional group at 3387 cm^{-1} and olefinic (C=C) absorption at 1649 cm^{-1}. The GC-MS spectrum of **1** revealed a molecular ion peak at m/z 414 $[M]^+$. The ^{1}H NMR spectrum displayed six methyl signals at δ_H 0.72 (3H, s, H-18), 0.86 (3H, t, *J* = 6.5 Hz, H-29), 0.93 (6H, d, *J* = 6.5 Hz, H-26/27), 0.97 (3H, d, *J* = 6.5 Hz, H-21) and 1.06

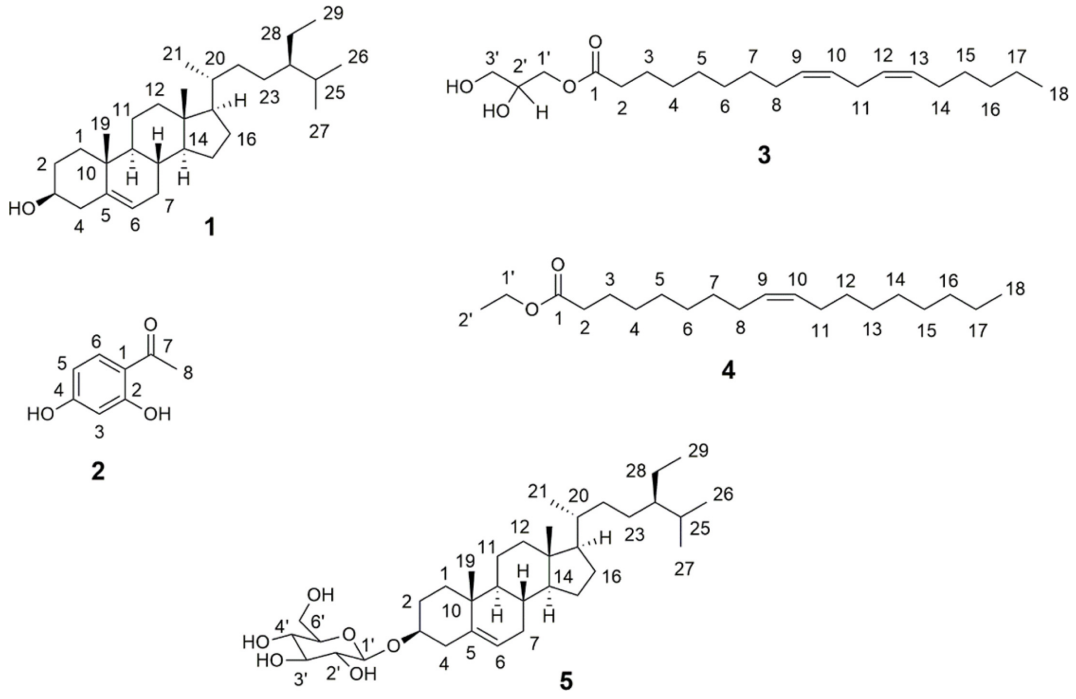

Fig. 1. Structures of the isolated compounds from stem-bark of *A. occidentale.*

(3H, s, H-19). Oxymethine signal appeared δ 3.54 (m, H-3) and olefinic double bond was revealed by the signal at δ_H 5.35 (, 1H, bd J = 5.0 Hz, H-6). The ^{13}C NMR showed methyl signals at δ_C 11.8 (C-29), 12.0 (C-18), 18.8 (C-19), 19.0 (C-21), 19.4 (C-26) and 19.8 (C-27); oxymethine signal at δ_C 71.7 (C-3) and olefinic carbon signals at δ_C 121.7 (C-6) and 140.7 (C-5). Based on the above assignment and the comparison of IR, MS, ^{1}H, ^{13}C NMR spectral data with those reported in the literature,[22] the structure of **1** was identified as β-sitosterol.

Compound **2** was obtained as off-white powder. The IR spectrum showed absorption bands for the presence of O-H group at 3418 cm^{-1} and carbonyl group at 1670 cm^{-1}. The ESI-MS of **2** showed a molecular ion peak at m/z 153 $[M+H]^+$. The ^{1}H NMR spectrum showed a methyl singlet at δ_H 2.60 (3H, s, H-8), three aromatic methine proton signals δ_H 6.88 (1H, d, J = 8.1 Hz, H-6), 7.52 (1H, s, H-3) and 7.45 (1H, d, J = 8.2 Hz, H-5). The ^{13}C NMR spectrum identified methyl signal at δ_C 23.4 (C-8), aromatic carbon signals at δ_C 115.7 (C-3), 117.5 (C-5), 123.6 (C-1), 134.6 (C-6), 167.7 (C-4), 168.9 (C-2) and the carbonyl carbon signal at δ_C 206.5. The spectroscopic data of **2** was carefully compared with literature data[23] and the compound was identified as 2,4-dihyroxyacetophenone.

Compound **3** was obtained as a colourless oil. The IR spectrum showed absorption bands for hydroxy and carbonyl groups at 3397 cm^{-1} and 1734 cm^{-1} respectively. The ESI-MS of **3** showed a molecular ion peak at m/z 377 $[M+Na]^+$. The ^{1}H NMR spectrum of 3 revealed a methyl signal at δ_H 0.87 (3H, t, J = 6.5 Hz, H-18), seven methylene signals in the upfield region at δ_H 1.25 (14H, m, H-4, H-5, H-6, H-7, H-15, H-16, H-17), a mildly deshielded methylene signal at δ_H 1.60 (2H, m, H-3), three allylic methylene signals at δ_H 2.03 (6H, m, H-8, H-11, H-14), and a methylene signal bonded to carbonyl group at δ_H 2.33 (2H, t, J = 7.2 Hz, H-2). Five oxygenated proton signals appeared at δ_H 3.59-4.15 (5H, m, H-1′, H-2′, H-3′) and the four olefinic protons were identified at δ_H 5.34 (4H, m, H-9, H-10, H-12, H-13). The ^{13}C-NMR spectrum showed the presence of methyl signal at δ_C 14.0 (C-18), eight methylene signals at δ_C 29.0-29.7 (C-3, C-4, C-5, C-6, C-7, C-15, C-16, C-17), three allylic methylene signals at δ 22.6-27.1 (C-8, C-11, C-14), four oxygenated carbon signals at δ_C 63.2 (C-1′), 65.0 (C-3′), and 70.1 (C-2′). Olefinic carbon signals appeared at δ_C 129.6-129.9 (C-9, C-10, C-12, C-13). Based on the available spectroscopic data, the compound was identified as 1-monolinolein. The authenticity of the data was confirmed by comparison with literature data.[24]

Compound **4** was obtained as a colourless oil. Compound **4** showed IR absorption band for carbonyl group at 1694 cm^{-1}. The GC-MS of **4** showed a molecular ion peak at m/z 310 $[M]^+$ The ^{1}H NMR spectrum of 4 showed two methyl signals at δ_H 0.88 (3H, t, J = 6.5 Hz, H-18) and 1.24 (3H, t, J = 7.0 Hz, H-2′). Ten methylene signals appeared at δ_H 1.25-1.29 (20H, m, H-4, H-5, H-6, H-7, H-12, H-13, H-14, H-15, H-16, H-17) and one methylene signal at slightly downfield region at δ_H 1.61 (2H, m, H-3). Two allylic methylene signals appeared at δ_H 2.01 (4H, q, J = 6.5 Hz, H-8, H11) and the presence of methylene adjacent to carbonyl group was identified at δ_H 2.27 (2H, t, J = 7.5 Hz, H-2). Besides, oxygenated and olefinic proton signals showed at δ_H 4.13 (2H, q, J = 7.0 Hz, H-1′) and δ_H 5.34 (2H, m, H-9, H-10) respectively. The ^{13}C NMR spectrum revealed twenty carbon signals including carbonyl carbon signal at δ_C 173.9 (C-1), olefinic carbon peaks at δ_C 129.7 (C-9) and 130.0 (C-10). Beside allylic and oxygenated carbon peaks at δ_C 27.2 (C-8, C-11) and 60.1 (C-1′), two methyl carbon signals showed at δ_C 14.1 (C-18), 14.3 (C-2′), twelve methylene carbon signals showed at δ_C 22.7 (C-17), 25.0 (C-3), 29.0-29.8 (C-4, C-5, C-6, C-7, C-12, C-13, C-14, C-15), 31.9 (C-16), and 34.4 (C-2). In the HMBC spectrum, the correlations of δ_H 2.01 (H-8, H-11) with δ_C 129.7, 130.0 (C-9, C-10) and δ_H 1.61 (H-3) with δ_C 173.9 (C-1) were observed. The double bond geometry was in the Z configuration according to the chemical shift of the allylic methylenes (δ_C 27.20, C-8, C-11).[25] The ^{13}C-NMR spectral data of this unsaturated fatty ester was in good agreement with those of elaidic acid.[26,27] Hence, the structure of compound **4** was identified as ethyl oleate.

Compound **5** was obtained as an amorphous powder. The IR spectrum revealed the presence of hydroxyl group at 3419 cm^{-1}, olefinic group at 1633 cm^{-1} and ether group at 1023 cm^{-1}. The HR-ESIMS of **5** gave an intense parent ion at m/z 599.429 $[M+Na]^+$ corresponding to the molecular formula of $C_{35}H_{60}O_6Na$ which requires six degrees of unsaturation. The ^{1}H-NMR spectrum of **5** displayed one primary methyl signal at δ_H 0.88 (3H, t, J = 7.4 Hz, H-29), three secondary methyl signals at δ_H 0.86 (3H, d, J = 7.0 Hz, H-27), 0.91 (3H, d, J = 7.4 Hz, H-26), 1.02 (3H, d, J = 6.5 Hz, H-21) and two quaternary methyl signals at δ 0.69 (3H, s, H-18) and 0.95 (3H, s, H-19) which are typical of stigmastane steroidal methyl groups. Besides, one oxymethine proton at δ_H 3.87 (1H, m, H-3) and one olefinic proton peak at δ_H 5.37 (1H, m, H-6) together with one anomeric proton signal at δ_H 5.06 (1H, d, J = 7.6 Hz, H-1′) were recognized. The ^{13}C-NMR spectrum of 5 revealed thirty-five carbon signals and the six carbon signals at δ_C 101.5, 79.4, 76.9, 76.3, 70.5 and

Table 1. IC_{50} values (μM) of compounds

	Cell lines		
Compounds	A549	SCOV3	NRK-49f
1	52.28	> 100	>100
2	> 100	> 100	> 100
3	> 100	> 100	> 100
5	> 100	> 100	> 100
Doxorubicin	2.5	4.06	6.2

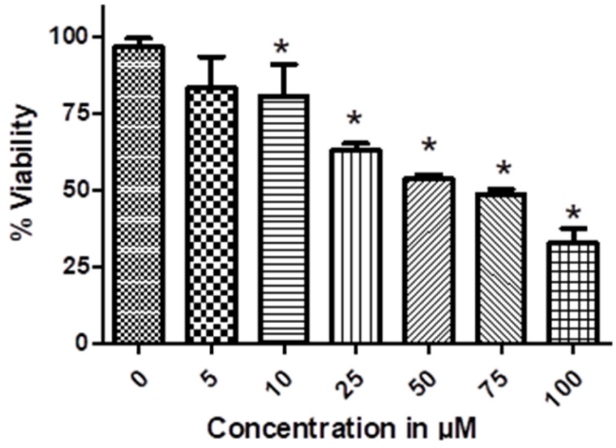

Fig. 2. Viabilities of A549 cancer cell line following treatment with compound **1**.

62.1 were recognized as typical of glucose moiety. The carbon signals at δ_C 79.3, 122.4 and 140.7 were recognized as the oxymethine and olefinic carbon signals of steroid aglycone moiety. From the above data, compound **5** was identified as β-sitosterol-3-O-β-D-glucopyranoside by comparison with literature data.[28]

The cytotoxicity activities of compounds **1-3** and **5** were tested on A549 (lung adenocarcinoma), SCOV3 (ovarian carcinoma) and NRK49f (normal rat kidney fibroblast) cell lines using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, compared with doxorubicin as the positive control (Table 1). Compound **1** showed moderate cytotoxicity activity (IC_{50} 52.28 μM) against human lung adenocarcinoma (A549). Meanwhile, compounds 2, 3 and 5 were inactive against the cell lines achieving $IC_{50} > 100$ μM. The criterion for cytotoxicity of crude extract was established by the U.S. National Cancer Institute (NCI) is an $IC_{50} < 20$ μM.[29] The cytotoxicity of compound 1 was observed in a dose-dependent and the cell viability above 10 μM was significantly different ($\rho < 0.05$) (Fig. 2).

In conclusion, *Anacardium occidentale* has been extensively reported for its chemical diversity. In this study, we have reported the isolation of five compounds of which four of them, compounds **2-5** were reported for the first time from this plant. Meanwhile, compound **1** showed a moderate antiproliferative activity against human lung adenocarcinoma A549 in a dose-dependent manner it is therefore considered to be a promising compound for further study on other human cancer cell lines. Thus, our finding has provided experimental data supporting the use of *A. occidentale* in traditional medicines.

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Conflicts of Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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