A New Triterpene from *Atalantia retusa* Merr.

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Atalantia retusa Merr. is a rare Philippine endemic shrub which was reported to exhibit high anti-nociceptive and anti-inflammatory activities. The dichloromethane extract of the air-dried leaves of *A. retusa* afforded a new triterpene, retusenol (**1**), and friedelin (**2**), dischidiol (**3**), 5,7-dimethoxy-8-(3-methyl-2-oxybutyl)coumarin (**4**), humulene (**5**), and β-caryophyllene (**6**). The structures of **1** – **4** were elucidated by extensive 1D and 2D NMR experiments. Friedelin, a known analgesic and anti-inflammatory drug, is an active principle of the shrub.

Compounds **1** – **4** were tested for cytotoxicity against the human cancer lung adenocarcinoma A549, colon carcinoma HCT116 and the non-cancer Chinese hamster ovary AA8 using the MTT assay. Triterpenes **1** – **3** had no linear interpolation with HCT 116 and A549, thus the IC_{50} value could not be computed. This implied that these compounds did not exhibit any cytotoxic effect against these cell lines. Meanwhile, **4** exhibited moderate cytoxicity against A549, HCT 116 and AA8 with IC_{50} values of 47.5634, 42.4338 and 46.2751 μg mL^{-1}, respectively. Compounds **1**, **2** and **4** were tested for their antimicrobial properties against seven microorganisms and exhibited the highest activity against *B. subtilis*, even surpassing the activity of the standard antibiotic Chloramphenicol. They also exhibited antimicrobial activities against *P. aeruginosa, S. aureus, C. albicans*, and *T. mentagrophytes*, but were inactive against *A. niger* and *E. coli.*

Key words: Rutaceae, Retusenol, Friedelin, Dischidiol

Introduction

Atalantia retusa Merr. (syn. *Severinia retusa* (Merr.) Swingle) is a wild member of the Citrus family (Rutaceae) that is endemic to Mindoro, Palawan and Panay islands in the Philippines. This rare shrub grows to about 3 meter high in thickets at low altitudes. It is locally known as tulan manok on Ilin Island, Mindoro, where the specimen for this study was collected. This species is apparently related to the more widespread Philippine endemic *Atalantia disticha* (Blanco) Merr., differing only in its leaves which are broad at both ends and in the flower characters [1].

There is no reported chemical study on the plant. A recent study referred to the high anti-nociceptive and anti-inflammatory activities of the hexane extract from the leaves of *A. retusa* [2]. This study was conducted to identify the non-polar constituents of the leaves of the plant which may be responsible for its anti-nociceptive and anti-inflammatory activities.

We report herein the isolation and structure elucidation of a new triterpene, retusenol (**1**), from the dichloromethane extract of the air-dried leaves of *A. retusa*. Friedelin (**2**), dischidiol (**3**), 5,7-dimethoxy-8-(3-methyl-2-oxybutyl)coumarin (**4**), humulene (**5**), and β-caryophyllene (**6**) were also isolated from leaves of the shrub (Fig. 1). We likewise report the cytotoxicity test results of **1** – **4** and on the antimicrobial activities of **1**, **2** and **4**.

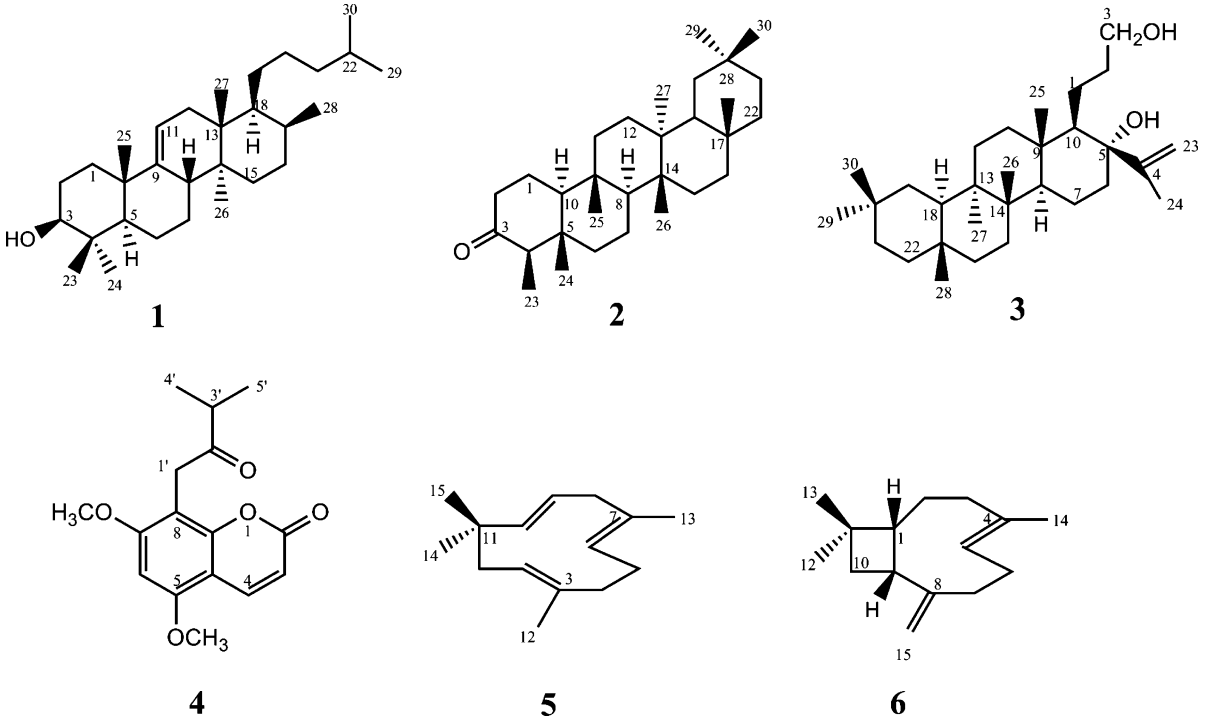

Fig. 1. Chemical constituents of *Atalantia retusa*: a new triterpene, retusenol (**1**), friedelin (**2**), dischidiol (**3**), 5,7-dimethoxy-8-(3-methyl-2-oxybutyl)coumarin (**4**), humulene (**5**), and β-caryophyllene (**6**).

Experimental Section

General experimental procedures

Optical rotations were measured with a Jasco DIP-370 digital polarimeter. IR spectra were recorded on a Perkin-Elmer 1600 Fourier Transform IR spectrometer. UV spectra were recorded on a U-2000 Hitachi UV/Vis spectrometer. The HR-EIMS was obtained on a Finnigan/Thermo Quest MAT 95 XL spectrometer. NMR spectra were recorded on a Varian VNMRS spectrometer in $CDCl_3$ at 600 MHz for ^{1}H NMR and 150 MHz for ^{13}C NMR spectra. Column chromatography was performed with silica gel 60 (70 – 230 mesh), while TLC was performed with plastic-backed plates coated with silica gel F_{254}. The plates were visualized with vanillin-H_2SO_4 and warming.

Plant material

The leaves of *Atalantia retusa* Merr. were collected from Barangay Ipil, Ilin Island, Occidental Mindoro, Philippines, in December 2009. Specimens of the plant were collected and authenticated by one of the authors (E. H. M.). A voucher specimen was deposited at the Biology Department, De La Salle University, Manila/Philippines under the number # 893.

Isolation of constituents from the leaves of A. retusa

The air-dried leaves (502 g) of *A. retusa* were ground, soaked in dichloromethane for three days and then filtered. The filtrate was concentrated under vacuum to afford the crude extract (16.5 g). The crude extract was fractionated by silica gel chromatography using increasing proportions of acetone in dichloromethane (10 % increments) as eluents. The fractions were collected and monitored by thin-layer chromatography (TLC). Fractions with spots of the same R_f value were combined and rechromatographed in appropriate solvent systems until TLC-pure isolates were obtained.

The crude dichloromethane extract of the leaves of *A. retusa* was chromatographed in increasing proportions of acetone in dichloromethane (10 % increments) as eluents. The DCM fraction was rechromatographed (10×) in petroleum ether to afford **5** (6 mg) and **6** (9 mg). The 10 % acetone in dichloromethane fraction was rechromatographed (6×) in 5 % ethyl acetate in petroleum ether to afford **2** (25 mg). The 20 % acetone in DCM fraction was rechro-

matographed (7×) in 10% ethyl acetate in petroleum ether to afford **1** (15 mg). The 30% acetone in DCM fraction was rechromatographed (5×) in diethyl ether-acetonitrile-DCM in a 0.5 : 0.5 : 9 ratio (v/v) to afford **4** (10 mg). The 40% acetone in DCM fraction was rechromatographed (6×) in diethyl ether-acetonitrile-DCM (0.5 : 0.5 : 9, v/v) to afford **3** (8 mg).

Cytotoxicity tests

Compounds **1**–**4** were tested for cytotoxic activity against a human colon carcinoma (HCT116) cell line, a human lung non-small cell adenocarcinoma (A549) cell line and the non-cancer cell line Chinese hamster ovary cells (AA8) at the Institute of Biology, University of the Philippines, Diliman, Quezon City/Philippines. All cell lines were obtained from the American Type Culture Collection (ATCC, Manassa Va./USA). Doxorubicin was used as the positive control, while dimethylsulfoxide (DMSO) was used as the negative control. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay reported in the literature was employed [3–6].

Antimicrobial assays

The microorganisms used in these tests were obtained from the University of the Philippines Culture Collection (UPCC). These are *Pseudomonas aeruginosa* (UPCC 1244), *Bacillus subtilis* (UPCC 1149), *Escherichia coli* (UPCC 1195), *Staphylococcus aureus* (UPCC 1143), *Candida albicans* (UPCC 2168), *Trichophyton mentagrophytes* (UPCC 4193), and *Aspergillus niger* (UPCC 3701). Compounds **1**–**3** were tested for antimicrobial activity against these microorganisms. The test compound (30 mg) was dissolved in 95% ethanol. The positive control for the bacteria is a Chloramphenicol sample (HiMedia Laboratories, Ltd.) which contains 30 mg Chloramphenicol in a 6 mm disc. The positive control for the fungi is Canesten (Bayer) which contains 1% Clotrimazol. The antimicrobial assay procedure reported in the literature [7] was employed. The clearing zone was measured in millimeters, and the average diameter of the clearing zones was calculated. The diameter of the well for the test compounds was 10 mm. The activity index was computed by subtracting the diameter of the well from the diameter of the clearing zones divided by the diameter of the well, *e. g.* activity index (AI) (diameter of clearing zone-diameter of well)/diameter of well.

*Retusenol (**1**)*

Colorless solid, m. p. 145 °C. – $[\alpha]_D = +57.6$ (c = 0.40, $CHCl_3$). – IR (neat): ν = 3380 (OH), 1133 (C-O), 2927, 2867, 1461, 1371 cm^{-1}. – ^1H and ^{13}C NMR: see Table 1. HRMS ((+)-EI): m/z = 428.4025 (calcd. 428.4013 for $C_{30}H_{52}O$, $[M]^+$).

*Friedelin (**2**)*

Colorless solid. – ^1H NMR (600 MHz, $CDCl_3$): $\delta = 1.68$, 1.96 (H_2-1), 2.30, 2.38 (H_2-2), 2.24 (H-4), 1.28, 1.76 (H_2-6), 1.38, 1.48 (H_2-7), 1.40 (H-8), 1.52 (H-10), 1.20, 1.38 (H_2-11), 1.35 (H_2-12), 1.32, 1.54 (H_2-15), 1.36, 1.56 (H_2-16), 1.56 (H-18), 1.20, 1.38 (H_2-19), 1.26, 1.48 (H_2-21), 0.96, 1.48 (H_2-22), 0.86 (H_3-23, d, J = 7.2 Hz), 0.70 (H_3-24, s), 0.85 (H_3-25, s), 0.99 (H_3-26, s), 1.03 (H_3-27, s), 0.98 (H_3-28, s), 0.93 (H_3-29, s), 1.16 (H_3-30). – ^{13}C NMR (150 MHz, $CDCl_3$): δ = 22.3 (C-1), 41.5 (C-2), 213.3 (C-3), 58.2 (C-4), 42.1 (C-5), 41.3 (C-6), 18.2 (C-7), 53.1 (C-8), 37.4 (C-9), 59.5 (C-10), 35.6 (C-11), 30.5 (C-12), 39.7 (C-13), 38.3 (C-14), 32.4 (C-15), 36.0 (C-16), 30.0 (C-17), 42.8 (C-18), 35.3 (C-19), 28.2 (C-20), 32.8 (C-21), 39.2 (C-22), 6.8 (C-23), 14.6 (C-24), 17.9 (C-25), 20.3 (C-26), 18.7 (C-27), 32.1 (C-28), 35.0 (C-29), 31.8 (C-30).

*Dischidiol (**3**)*

^1H NMR (600 MHz, $CDCl_3$): δ = 1.42, 2.02 (H_2-1), 1.48, 1.78 (H_2-2), 3.63, 3.65 (H_2-3), 1.55, 1.66 (H_2-6), 1.58, 2.38 (H_2-7), 1.56 (H-8), 1.68 (H-10), 1.30 (H_2-11), 1.32 (H_2-12), 1.32 (H_2-15), 0.94, 1.50 (H_2-16), 1.54 (H-18), 1.18, 1.36 (H_2-19), 1.26, 1.46 (H-21), 1.36, 1.56 (H-22), 4.89 (H-23, t, J = 1.2 Hz), 5.14 (H-23, s), 1.92 (H_3-24, d, J = 1.2 Hz), 0.81 (H_3-25, s), 0.95 (H_3-26, s), 1.01 (H_3-27, s), 1.15 (H-28, s), 0.92 (H_3-29, s), 0.97 (H_3-30, s). – ^{13}C NMR (150 MHz, $CDCl_3$): δ = 20.6 (C-1), 34.2 (C-2), 61.6 (C-3), 149.7 (C-4), 76.6 (C-5), 20.9 (C-6), 42.3 (C-7), 53.5 (C-8), 39.2 (C-9), 60.2 (C-10), 34.4 (C-11), 30.3 (C-12), 39.6 (C-13), 38.4 (C-14), 32.5 (C-15), 39.3 (C-16), 29.9 (C-17), 42.7 (C-18), 35.3 (C-19), 28.1 (C-20), 32.8 (C-21), 36.0 (C-22), 114.5 (C-23), 23.5 (C-24), 15.5 (C-25), 20.2 (C-26), 18.8 (C-27), 32.2 (C-28), 35.0 (C-29), 31.8 (C-30).

*5,7-Dimethoxy-8-(3-methyl-2-oxybutyl)coumarin (**4**)*

^1H NMR (600 MHz, $CDCl_3$): δ = 6.10 (H-3, d, J = 9.6 Hz), 7.96 (H-4, d, J = 9.6 Hz), 6.30 (H-6, s), 3.89 (H_2-1′, s), 2.77 (H-3′, sept, J = 6.6 Hz), 1.17 (H_3-4′ and H_3-5′, d, J = 6.6 Hz). – ^{13}C NMR (150 MHz, $CDCl_3$): δ = 161.3 (C-2), 110.8 (C-3), 138.8 (C-4), 103.7 (C-4 a), 156.2 (C-5), 90.1 (C-6), 161.2 (C-7), 104.7 (C-8), 153.9 (C-8a), 34.3 (C-1′), 211.3 (C-2′), 40.7 (C-3′), 18.4 (C-4′ and C-5′).

*Humulene (**5**)*

Colorless oil. – ^{13}C NMR (150 MHz, $CDCl_3$): δ = 42.0 (C-1), 124.9 (C-2), 133.1 (C-3), 39.7 (C-4), 23.3 (C-5), 125.8 (C-6), 139.2 (C-7), 40.2 (C-8), 127.7 (C-9), 141.0 (C-10), 37.3 (C-11), 27.2 (C-12, C-13), 17.3 (C-14), 15.0 (C-15).

Table 1. ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) data, and COSY, HMBC and NOESY correlations of **1** in $CDCl_3$.

Position	δ_C	δ_H, mult.[a] (*J* in Hz)	COSY	HMBC	NOESY
1	36.1	1.45, 1.78	H_2-2		
2	27.8	1.66, 1.74	H_2-1, H-3		
3	78.9	3.20, dd (11.4, 4.2)	H_2-2	H_3-23, H_3-24	H_3-24
4	39.1				
5	52.5	0.88	H_2-6	H_3-23, H_3-24, H_3-25	H_3-24, H_3-26
6	21.4	1.46, 1.68	H-5, H_2-7		
7	28.1	1.33, 1.66	H_2-6, H_2-8		
8	41.8	2.16	H_2-7	H_3-26	H_3-25, H_3-27
9	148.5			H-8, H-11, H_3-25	
10	39.4				
11	115.0	5.20	H_2-12		
12	37.1	1.90, 2.06	H-11		
13	44.3			H_3-26, H_3-27	
14	47.0			H_3-26, H_3-27	
15	33.9	1.35 (2H)	H_2-16		
16	36.5	0.98, 1.35	H_2-15, H-17		
17	28.0	1.52	H_3-28, H-18	H_3-28	
18	51.0	1.58, br d (6.0)	H-17, H_2-19	H_3-27, H_3-28	
19	28.02	1.62, 1.64	H-18, H_2-20		
20	29.1	1.14, 1.36	H_2-19, H_2-21		
21	39.5	1.13 (2H)	H_2-20	H_3-29, H_3-30	
22	36.1	1.46	H_3-29, H_3-30	H_3-29, H_3-30	
23	15.6	0.80 Me, s		H_3-24	H_3-25
24	28.2	0.97 Me, s			H-3, H-5
25	22.3	1.02 Me, s			H-8, H_3-23
26	18.5	0.72 Me, s			H-18
27	14.4	0.63 Me, s		H_2-12, H-18	H_3-28
28	18.4	0.85 Me, d (6.6)	H-17		H_3-27
29	22.5	0.84 Me, d (6.0)	H-22	H-22, H_3-30	
30	22.8	0.86 Me, d (6.0)	H-22	H-22, H_3-29	

[a] Multiplet unless otherwise indicated.

*β-Caryophyllene (**6**)*

Colorless oil. – ^{13}C NMR (150 MHz, $CDCl_3$): $\delta = 53.5$ (C-1), 29.3 (C-2), 39.9 (C-3), 135.5 (C-4), 124.3 (C-5), 28.3 (C-6), 34.8 (C-7), 154.7 (C-8), 48.5 (C-9), 40.3 (C-10), 33.0 (C-11), 22.7 (C-12), 30.1 (C-13), 16.3 (C-14), 111.6 (C-15).

Results and Discussion

Chromatographic separation of the dichloromethane extract of the air-dried leaves of *A. retusa* afforded a new triterpene, retusenol (**1**). The structure of **1** was elucidated by extensive 1D and 2D NMR spectroscopy as follows.

The ^{1}H NMR spectrum of **1** (Table 1) displayed isopropyl methyl doublets at $\delta = 0.84$ (d, $J = 6.0$ Hz) and 0.86 (d, $J = 6.0$ Hz), another methyl doublet at $\delta = 0.85$ (d, $J = 6.6$ Hz), five methyl singlets at $\delta = 0.63$, 0.72, 0.80, 0.85, 0.97, and 1.02, an olefinic proton signal at $\delta = 5.20$, and an oxymethine proton signal at $\delta = 3.20$. The ^{13}C NMR and DEPT spectra of **1** (Table 1) showed resonances for thirty carbon atoms with the following functionalities: an oxymethine carbon at $\delta = 78.9$ and olefinic carbons at $\delta = 148.5$ and 115.0; eight methyl, ten methylene, six methine and three quaternary carbons. These resonances suggested a triterpene skeleton with an olefin and an alcohol functionality.

The HREIMS of **1** gave a molecular ion of $m/z = 428.4025$ $[M]^+$, which corresponded to a molecular formula of $C_{30}H_{52}O$ indicating an index of hydrogen deficiency of five. With one olefin, the compound is tetracyclic.

The COSY spectrum of **1** (Table 1) indicated four isolated spin systems: H_2-1/H_2-2/H-3; H-5/H_2-6/H_2-7/H-8; H-11/H_2-12; H_2-15/H_2-16/H-17/H_3-28, H-18/H_2-19/H_2-20/H_2-21/H-22/H_3-29, H_3-30 (Fig. 2).

The protons attached to carbon atoms were assigned from HSQC 2D NMR data (Table 1), and the structure

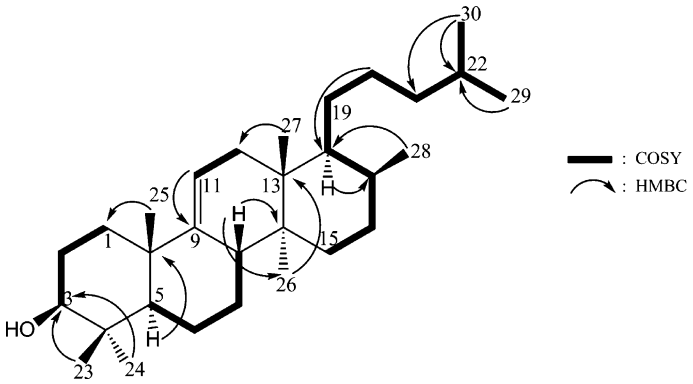

Fig. 2. ^{1}H-^{1}H COSY and key ^{1}H-^{13}C long-range correlations of **1**.

of **1** was elucidated by analysis of the HMBC 2D NMR data (Table 1). Key HMBC correlations are shown in Fig. 2. Thus, the oxymethine carbon was assigned to C-3 on the basis of long-range correlations to the methyl singlets at H_3-23 and H_3-24. The double bond was assigned to C-9 due to long-range correlations to H-11, H-8 and H_3-25. The isopropyl methyl protons were attributed to H_3-29 and H_3-30 since long-range correlations were observed between these protons and C-22 and C-21. The methyl doublet H_3-28 was attached to C-17 based on the long-range correlations between these protons and C-17. All long-range correlations are consistent with the structure of **1**.

The relative stereochemistry of **1** was deduced from the NOESY spectrum (Table 1). The carbinyl proton |H-3| which was close in space to the methyl protons |H_3-24|, the methine proton |H-5|, which was in turn close to |H_3-26|, and which was finally close to the methine proton |H-18|, indicated that these groups are on the same face of **1**. On the opposite face of the molecule, the methyl group of doublet |H_3-28|was close in space to the methyl group singlet of |H-27|, which was in turn close to methine proton |H-8|, which was also close to methyl group of singlet |H_3-25|, which was finally close to another methyl singlet |H_3-23|. Thus, **1** has the relative configuration shown in Fig. 3. The trivial name retusenol is suggested for **1**.

The structure of **2** was elucidated by extensive 1D and 2D NMR spectroscopy and confirmed by comparison of its ^{13}C NMR data (see Experimental Part) with those of friedelin [8]. The structure of **3** was elucidated by extensive 1D and 2D NMR spectroscopy (see Experimental Part). This compound was first reported as a constituent of *Dischidia formosana* [9]. The structures of **4** – **6** were confirmed by

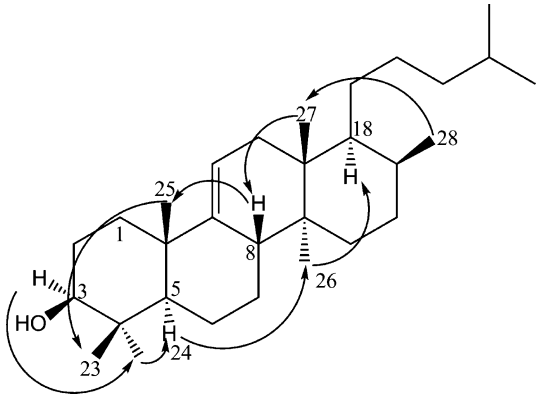

Fig. 3. Key NOESY correlations of **1**.

comparison of their ^{13}C NMR data (see Experimental Part) with those of 5,7-dimethoxy-8-(3-methyl-2-oxybutyl)coumarin (**4**) [10], humulene (**5**) [11] and β-caryophyllene (**6**) [12].

A literature search revealed that a European Patent [13] provides an analgesic/anti-inflammatory drug which comprises quercetin-3-*O*-glucoside or a friedelan-type compound (friedelin, friedelan-3-α-ol or friedelan-3-β-ol) from the alcohol extract of *Maytenus ilicifolia* as an effective component. Another study reported that friedelin at 40 mg kg^{-1} inhibited the acute phase of inflammation in Wistar rats with maximum inhibitions of 52.5 % and 68.7 % ($P < 0.05$) in carrageenan-induced paw edema and croton oil-induced ear edema, respectively. Friedelin also produced significant ($P < 0.05$) analgesic activity in the acetic acid-induced abdominal constriction response and formalin-induced paw licking response in Wistar mice [14]. Thus the high anti-nociceptive

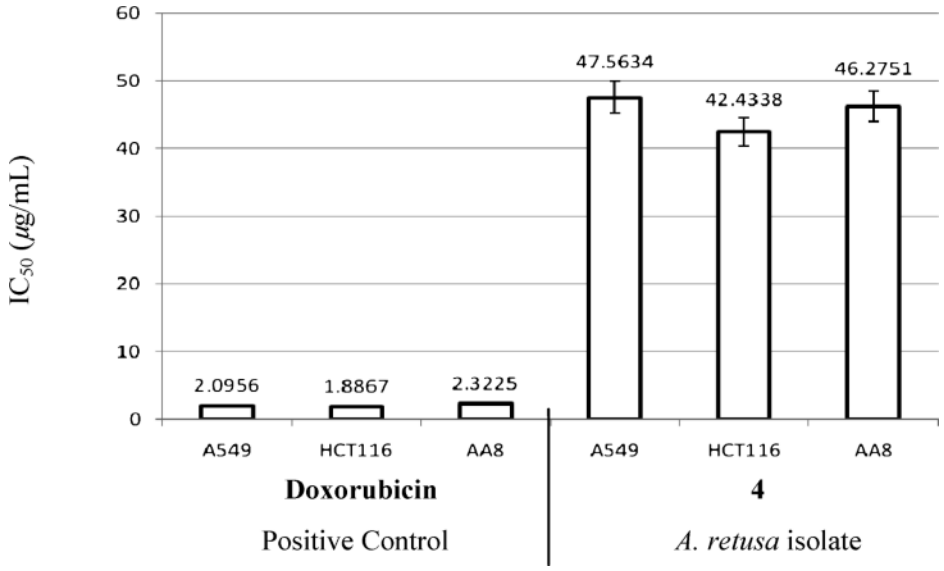

Fig. 4. Inhibitory concentrations at 50% (IC_{50}) of **4** from *A. retusa* tested against human cancer cell lines using the MTT assay: lung adenocarcinoma A549, colon carcinoma HCT116 and the non-cancer Chinese hamster ovary AA8. Each value is the mean of three trials with three replicates per trial with SD indicated by bars.

and anti-inflammatory activities of the hexane extract from the leaves of *A. retusa* may be attributed to friedelin.

Cytotoxicity tests on **1**–**4** indicated that **4** exhibited moderate cytoxicity against A549, HCT 116 and AA8 with IC_{50} values of 47.5634, 42.4338 and 46.2751 $\mu g\,mL^{-1}$, respectively (Fig. 4). Triterpenes **1**–**3** had no linear interpolation with HCT 116 and A549, and thus the IC_{50} values could not be computed. This implied that these compounds did not exhibit cytotoxic effects against these cell lines.

Compounds **1**, **2** and **4** were tested for antimicrobial properties against seven microorganisms (Table 2). The compounds exhibited the highest activity against *B. subtilis* with an activity index AI > 4.5, even surpassing the activity of the standard antibiotic Chloramphenicol with an AI value of 2.3. They also exhibited antimicrobial activities against *P. aeruginosa* with AI values of 0.7, 0.6 and 0.4, respectively, against *S. aureus* with AI values of 0.5, 0.5 and 0.6, respectively, against *C. albicans* with AI values of 0.4, 0.3 and 0.4, respectively, and against *Trichophyton mentagrophytes* with AI values of 0.3, 0.5 and 0.5, respectively. Compounds **1** and **2** were slightly active against *E. coli* with AI values of 0.2 and 0.1, respectively, while **4** was inactive against this microorganism. All the compounds tested were inactive against *A. niger*.

A recent study reported that friedelin exhibited moderate antifungal activity against *T. mentagro-*

Table 2. Antimicrobial test results of **1**, **2** and **4**.

Microorganism	Compounds	Clearing zone[a], mm	Activity index (AI)
Pseudomonas aeruginosa	**1**	17	0.7
	2	16	0.6
	4	14	0.4
	Chloramphenicol	14	1.3
Staphylococcus aureus	**1**	15	0.5
	2	15	0.5
	4	16	0.6
	Chloramphenicol[c]	25	3.2
Escherichia coli	**1**	12	0.2
	2	11	0.1
	4	–[b]	0
	Chloramphenicol[c]	23	2.8
Bacillus subtilis	**1**	> 55	> 4.5
	2	> 55	> 4.5
	4	> 55	> 4.5
	Chloramphenicol[c]	20	2.3
Candida albicans	**1**	14	0.4
	2	13	0.3
	4	14	0.4
	Canesten, 0.2 g[d]	18	0.8
Trichophyton mentagrophytes	**1**	13	0.3
	2	15	0.5
	4	15	0.5
	Canesten, 0.2 g[d]	55	4.5
Aspergillus niger	**1**	–[b]	0
	2	–[b]	0
	4	–[b]	0
	Canesten, 0.2 g[d]	23	1.3

[a] Average of 3 replicates; [b] no inhibition growth of test organism; [c] Chloramphenicol disc – 6 mm diameter; [d] contains 1% clotrimazol.

phytes, *Scopulariopsis* sp, *T. rubrum* 57/01, and *C. albicans* with an MIC value of $> 250\,\mu g\,mL^{-1}$, and *T. sinii, E. floccosum, A. niger* and *Magnethophora* sp with an MIC value of $125\,\mu g\,mL^{-1}$, and good activity against *T. rubrum* 296 and *C. lunata* with an MIC value of $62.5\,\mu g\,mL^{-1}$ [15]. In another study, friedelin was reported as inactive against the gram-positive bacteria: *B. cereus, S. aureus, S. saprophytes*, and *S. agalactinae*, gram-negative bacteria: *E. cloacae, E. coli, P. aeruginosa, S. mirabilis*, and *S. typhimurium* and the yeasts *C. albicans* and *C. tropicalis* with an MIC value of $> 1000\,\mu g\,mL^{-1}$ [16]. An earlier study referred to friedelin as active against the following bacteria with zones of inhibitions in parenthesis: *S. aureus* (6.60 mm), *S. typhi* (3.53 mm), *K. pneumonia* (4.09 mm), *P. microbillis* (3.11 mm), and *C. diptheriae* (3.50 mm). It was inactive against *E. coli* [17].

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