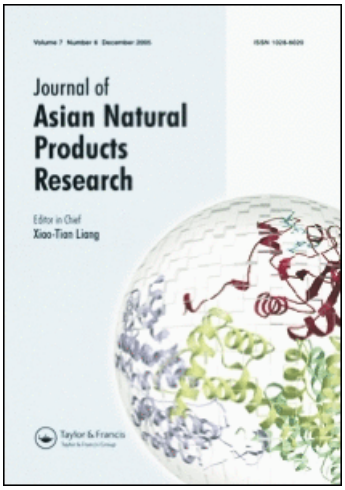

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Novel diterpenes with cytotoxic, anti-malarial and anti-tuberculosis activities from a brown alga *Dictyota* sp.

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Novel diterpenes with cytotoxic, anti-malarial and anti-tuberculosis activities from a brown alga *Dictyota* sp.

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Two new diterpenes, (1*E*,2*R**,3*R**,4*S**,6*E*,18*S**)-4,18-dihydroxydictyolactone (**1**) and 8α,11-dihydroxy-pachydictyol A (**2**), together with fucoxanthin (**3**) and a known diterpene 4α-hydroxycrenulatane (**4**) were isolated from the methanol extract of the brown alga *Dictyota* sp. collected from Bangsaen Beach, Thailand. Their structures were determined by spectroscopic data. The relative stereochemistry of **1** was established by NOE difference and NOESY experiments. Compound **1** showed weak anti-tuberculosis activity while **2** displayed strong cytotoxicity against the NCI-H187 cell line, and potent anti-malarial activity. In addition, **3** was active against HSV-1 and malarial parasites.

Keywords: *Dictyota*; Diterpene; NCI-H187; HSV-1; Anti-malarial

1. Introduction

Numerous chemical studies have indicated that brown seaweeds produce hundreds of secondary metabolites, including terpenoids (mostly sesquiterpenes and diterpenes), acetogenins, amino acid-derived compounds, fatty acids, sterols and carotenoids. Several seaweeds produce large numbers and types of secondary metabolites that exhibit a wide range of biological activities against diverse agents in the marine environment [1].

Brown algae of the genus *Dictyota* produce a significant number of secondary metabolites, especially diterpenes. Generally, these diterpenes have one of three types of carbon skeletons: xenicane, dolabellane, and extended sesquiterpene [2,3]. *Dictyota* secondary metabolites showed antiviral [2,4,5], cytotoxic [6] and anti-feedant activities [1,7]. In this study, we screened the crude extract of the brown alga *Dictyota* sp. collected from Bangsaen Beach, Thailand, for various biological activities. Bioactivity-guided fractionation led to the isolation of two new diterpenes, together with known compounds, carotenoid and crenulide, from the algal extract.

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2. Results and discussion

Compound **1** was isolated as yellow oil and its molecular formula was $C_{20}H_{30}O_4$ (HRESI-MS, *m/z* 335.2211 $[M + H]^+$). The ^{13}C NMR spectrum displayed 20 signals (table 1) whose multiplicities were determined by DEPT spectrum (four methyls, four methylenes, eight methines and four non-protonated carbons). The presence of seven sp^2 resonances in the ^{13}C NMR spectrum of **1** [δ 141.8 (C-9), 136.3 (C-6), 133.9 (C-1), 132.0 (C-14), 125.2 (C-7), 123.8 (C-13)], for three double bonds and an ester lactone carbonyl carbon [δ 172.5 (C-19)], accounted for four of the six degrees of unsaturation. This suggested that **1** is a bicyclic compound. The ^1H NMR spectrum of **1** showed three methyl singlets at δ 1.93 (H_3-20), 1.66 (H_3-16) and 1.56 (H_3-15), together with one methyl doublet at δ 1.08 (H_3-17). The signals at δ_C 72.6 and δ_H 4.32 (dd, $J = 2.0, 4.2$ Hz) (CH-4) and δ_C 98.0 and δ_H 5.88 (s) (CH-18) indicated the presence of two hydroxyl groups. One of them (CH-18) was connected with an ester lactone. The ^1H NMR spectrum also showed germinal coupling protons at δ 2.18, 2.36 ($^2J_{HH} = 12.7$ Hz) and 2.97, 3.27 ($^2J_{HH} = 17.5$ Hz). After assignment of each direct C—H bond by HMQC data, the partial structures —C_4—C_5—, —C_7—C_8—C_9— and C_{17}—C_{10}—C_{11}—C_{12}—C_{13}— were established by the coupling constant value and ^1H—^1H COSY analysis. The structure of **1** (figure 2) was established by HMBC correlations, as shown in figure 1.

Table 1. NMR data of **1** (400 MHz, $CDCl_3$).

No	δ_C	δ_H (*J*)	*COSY*	*HMBC*	*NOESY*	*NOE diff*
1	133.9 C	–	–	–	–	–
2	44.4 CH	3.19 s	–	C-1, C-3, C-4, C-10, C-18, C-19	H-18	H-18, H-20
3	50.1 CH	2.01 s	–	C-2, C-4, C-5, C-10, C-17, C-18, C-11, C-1	H-7, H-4	H-7
4	72.6 CH	4.32 dd ($J = 2.0, 4.2$)	H^1-5, H^2-5	C-2, C-6	H-17, H^1-5, H^2-5, H-3	H-17, H-3, H^1-5, H^2-5
5	49.0 CH_2	2.18 dd ($J = 4.2, 12.7$)	H^2-5, H-4	C-20	H-4, H-7	H-7, H-4, H^2-5
		2.36 d ($J = 12.7$)	H^1-5, H-4	C-3, C-4, C-7, C-6	H-4	H-4, H^1-5
6	136.3 C	–	–	–	–	–
7	125.2 CH	5.30 dd ($J = 3.9, 11.2$)	H^1-8, H^2-8	–	H^1-8, H-3, H^1-5	H-3, H^1-8, H^1-5
8	29.6 CH_2	2.97 ddd ($J = 4.8, 7.1, 17.5$)	H^2-8, H-7, H-9	–	H-7, H-9	H-9
		3.27 dtt ($J = 2, 11.4, 17.5$)	H^1-8, H-7, H-9		H-9	H-20
9	141.8 CH	7.01 tt ($J = 2.0, 7.3$)	H^1-8, H^2-8	C-2, C-7, C-8, C-19	H^1-8, H^2-8	H^1-8
10	32.5 CH	1.59 m	H-17, H-11	C-17	H-18	–
11	38.1 CH_2	1.21 m	H-10, H-12	C-10, C-12, C-13, C-17, C-3	–	–
12	25.8 CH_2	1.88 m	H-11, H-13	C-13	–	–
13	123.8 CH	5.00 tt ($J = 1.3, 7.1$)	H-12	C-12, C-15, C-16	–	–
14	132.0 C	–	–	–	–	–
15	17.7 CH_3	1.56 s	–	C-13, C-14, C-16	–	–
16	25.6 CH_3	1.66 s	–	C-13, C-14, C-15	–	–
17	18.2 CH_3	1.08 d ($J = 6.7$)	H-10	C-10, C-11, C-3	H-18, H-4	H-18, H-4
18	98.0 CH	5.88 s	–	C-1, C-2, C-3, C-19	H-17, H-2, H-10	H-2, H-17, H-10
19	172.5 C	–	–	–	–	–
20	20.1 CH_3	1.93 s	–	C-5, C-6, C-7	–	H^2-8, H-2, H^2-5

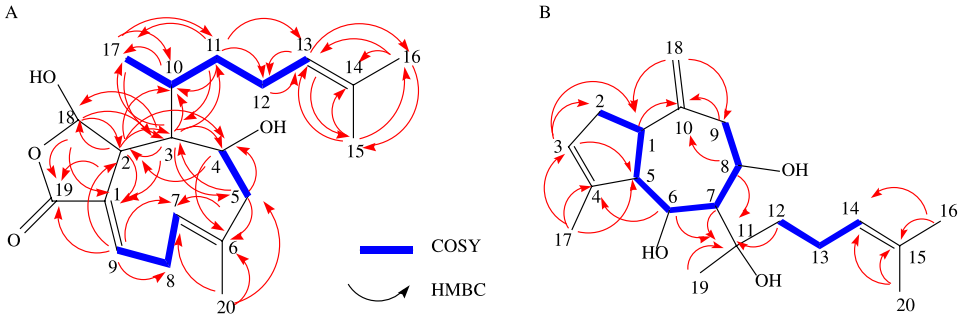

Figure 1. (a) $^{1}H—^{1}H$ COSY and HMBC correlations of **1**. (b) $^{1}H—^{1}H$ COSY and HMBC correlations of **2**.

The relative configuration of **1** was determined from NOESY cross peaks and NOE difference experiments. The key correlations between H-2 and H-8 (assigned for 2*R** and 18 *S**), H-3 and H-7, H-4 and CH_3-17, H-5, H-3 (assigned for 4*S**) were observed. Although H-10 was correlated to H-18, the C-10 bond can rotate. So C-10 could not be assigned. In addition, $C_6—C_7$ double bond was assigned to an *E* configuration since H-7 was opposite to CH_3-20. Similarly H-9 was on the opposite side to CH-2, as H-9 was correlated only to CH_2-8 by NOESY. Therefore, $C_1—C_9$ double bond was assigned to an *E* form. However, an attempt to deduce the absolute configuration of **1** by advance Mosher's method was unsuccessful due to decomposition of the MTPA esters soon after isolation. Hence **1** was elucidated as (1*E*,2*R**,3*R**,4*S**,6*E*,18*S**)-4,18-dihydroxydictyolactone. Though a similar type of compound, 4-acetoxydictyolactone was previously isolated from *Dictyota dichotoma* [8], to our knowledge, herein **1** was isolated from *Dictyota* sp. for the first time.

Diterpene **2** was isolated as a pale yellow powder with the molecular formula $C_{20}H_{32}O_3$ (HRESI-MS of $C_{20}H_{32}O_3Na$, *m/z* 343.2249 [M + Na^+]). The ^{13}C NMR spectrum displayed twenty signals (table 2), and their multiplicities were determined by the DEPT spectrum showing four methyls, five methylenes, seven methine and four quaternary carbons. There are five degrees of unsaturation in compound **2**. HMQC data of **2** assigned each proton to the corresponding carbon. $^{1}H—^{1}H$ COSY correlations established the partial structures $C_2—C_1—C_5—C_6—C_7—C_8—C_9$ and $C_{12}—C_{13}—C_{14}$. The structure of **2** (figure 2) was established by HMBC correlations, as shown in figure 1.

The relative configuration of **2** was determined by NOESY cross peaks and NOE difference experiments. The key correlations between H-1 and H-7 (assigned for 1*S**), H-5 and H-8 (assigned for 5*S** and 8*S**), H-6 and H-7 (assigned for 6*S** and 7*S**) were observed. In other words, H-1 was on the opposite side to H-5. But H-5 was on the same side to the hydroxyl group at C-6. H-6 was on the same side to H-7, but opposite to H-8 which was different from the known hydroazulenoid, 8β,11-dihydroxypachydictyol A [9]. In addition, H-3 was correlated to H-2 and CH_3-17. So double bond $C_3—C_4$ was *Z*-configuration. The configuration at C-11 could not be assigned as the C-11 bond can rotate. From this study **2** was different from 8β,11-dihydroxypachydictyol A isolated from the brown alga *Glossophora kunthii* [9] at C-8. The hydroxyl group at C-8 of **2** was on the same side to H-7. Therefore, **2** was 8α,11-dihydroxypachydictyol A. The configuration of **2** was 1*S**,3*Z*,5*S**,6*S**,7*S**,8*S**. Unfortunately, Mosher analysis of **2** was unsuccessful because of decomposition of the MTPA esters soon after isolation.

Table 2. NMR data of **2** (400 MHz, $CDCl_3$).

No.	δ_C	δ_H (*J*)	*COSY*	*HMBC*	*NOESY*	*NOE diff*
1	46.9 CH	2.64 q ($J = 9$)	H-2, H-5	C-5, C-10	H-7	H-7
2	34.3 CH_2	2.33 m	H-1	–	H-3, H^2-18	–
3	124.4 CH	5.34 s	–	C-1, C-2, C-5	H-17, H-2	–
4	141.1 C	–	–	–	–	–
5	58.9 CH	2.42 m	H-1, H-6	–	H-8	H-8, H^2-9
6	72.1 CH	4.27 dd ($J = 2.5, 6.0$)	H-7, H-5	C-4, C-5, C-7, C-8, C-11	H-7	H-7
7	52.8 CH	1.78 dd ($J = 2.5, 7.4$)	H-6, H-8	C-6, C-11, C-5, C-8	H-6	H-1, H-6
8	67.8 CH	4.35 td ($J = 2.6, 7.4$)	H-7, H-9	C-6, C-7, C-9, C-10, C-18	H-5, H-9	H-5, H-9, H-19
9	46.9 CH_2	2.48 dd ($J = 7.6, 14.6$)	H-9, H-8	C-1, C-7, C-8, C-18, C-10	H-18	–
		2.98 d ($J = 14.1$)	H-9, H-8	C-7, C-8, C-18, C-10	H-18, H-8	
10	147.6 C	–	–	–	–	–
11	77.2 C	–	–	–	–	–
12	41.4 CH_2	1.80 m	H-13	C-11, C-13, C-14	–	–
13	23.1 CH_2	1.98 br m	H-12, H-13, H-14	C-15, C-14	–	–
		2.10 br m	H-12, H-13, H-14	C-15, C-14		
14	124.1 CH	5.12 t ($J = 7.1$)	H-13	C-20, C-16	H-16	–
15	132.0 C	–	–	–	–	–
16	25.7 CH_3	1.70 s	–	C-14, C-15, C-20	H-14	–
17	15.0 CH_3	1.80 s	–	C-3, C-4, C-5	H-3	–
18	111.1 CH_2	4.87 s	–	C-1, C-9	H-2	–
		4.93 s	–		H-9	
19	27.5 CH_3	1.47 s	–	C-11, C-12, C-7	–	H-7, H-6, H-8
20	17.7 CH_3	1.62 s	–	C-14, C-15, C-16	–	–

Activity results of **1**, **2**, and **3** are displayed in table 3. Unfortunately, the diterpene **4** was isolated only 4.2 mg, therefore no bioactivity was obtained.

3. Experimental

3.1 General procedures

High-resolution ESI, APCI mass spectral data were recorded on micro TOF, Bruker at the Chulabhorn Research Institute. All NMR spectra were recorded using Bruker 400 AVANCE spectrometer at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR with $CDCl_3$ as solvent. Infrared spectra were recorded as thin films using a Perkin Elmer PARAGON 1000 spectrometer. Ultraviolet spectra were determined by Shimadzu UV-160 spectrometer with dichloromethane as solvent. Optical rotation measurements were recorded at 20°C on a Perkin Elmer 341 Polarimeter.

3.2 Plant material

The brown alga *Dictyota* sp. was intertidally collected from Hard-Wonnapa, Bangsaen Beach in October, 2003. It was kept frozen until analyzed. A voucher specimen (BI-MS-PA0218) is deposited at the Institute of Marine Science, Burapha University, Bangsaen,

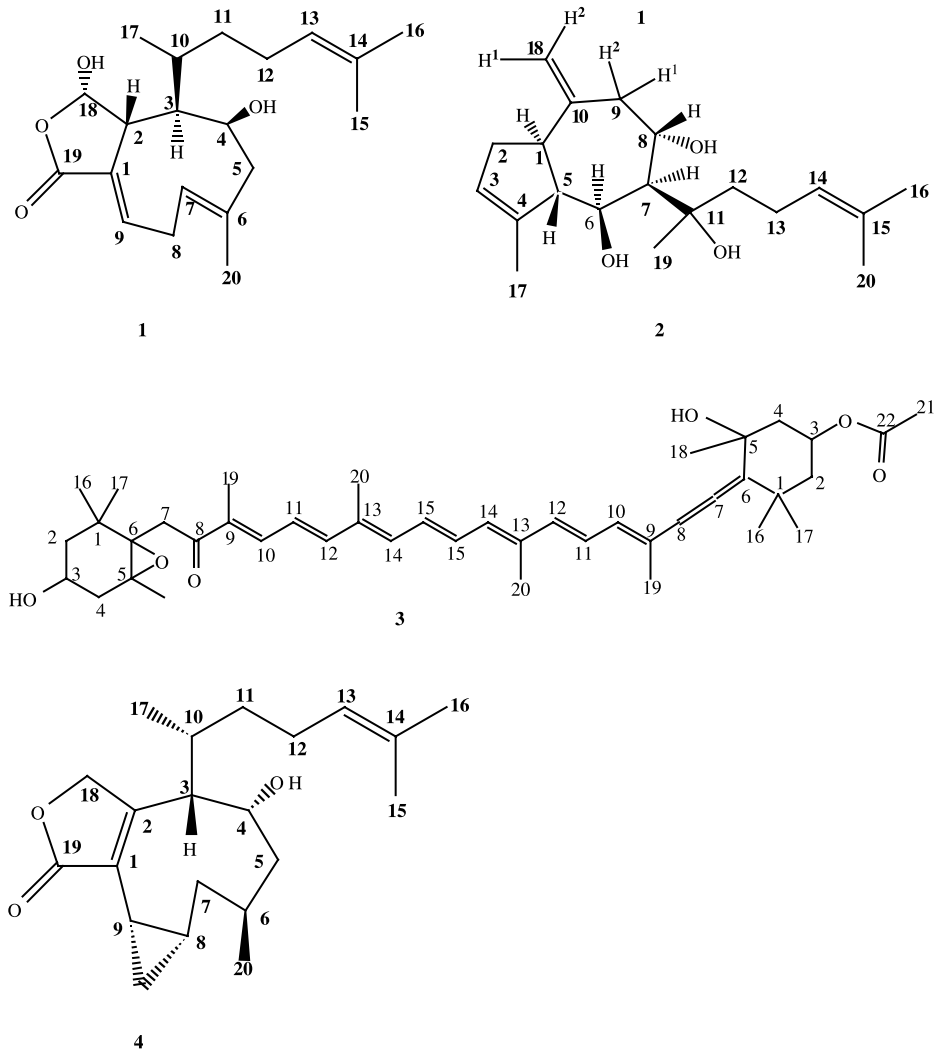

Figure 2. Chemical structures of compounds **1**, **2**, **3**, and **4**.

Chonburi. Unfortunately, the collected alga had incomplete sexual organs and therefore the species could not be identified.

3.3 Extraction and isolation

The fresh alga *Dictyota* sp. was extracted at room temperature with methanol. The organic residue was partitioned with dichloromethane and ethyl acetate to afford a dark oily extract (14.0478 g). The crude extract (see assay results in table 3) was subjected to a column chromatography on Si gel using hexane, dichloromethane, ethyl acetate and finally methanol as eluents. The ethyl acetate fraction (F3) (3.7716 g) was then subjected to the Si gel flash column chromatography using CH_2Cl_2, 50% EtOAc/CH_2Cl_2, EtOAc and MeOH as eluents to give five fractions (F3.1–F3.5).

Table 3. Biological activities of the *Dictyota* sp. extract and isolated compounds.

	Extract concentration having activity against the cell line, organism or enzyme (μg/ml)								
Type	*Vero*[†]	*HSV-1*[‡]	*KB*[¶]	*BC*[§]	*NCI*[‖]	*CA*[#]	*MT***	*PF*[††]	*COX*[‡‡]
Crude	13.1	0.700	2.17	2.27	1.90	–	6.25	2.90	–
1							200		
2			14.1		5.00			3.22	
3		5.00						2.90	

[†] Cytotoxicity towards Vero cells, IC_{50}.
[‡] *Herpes simplex* virus type 1, IC_{50}.
[¶] Oral carcinoma cell line, IC_{50}.
[§] Breast cancer cell line, IC_{50}.
[‖] Lung cancer cell line, IC_{50}.
[#] *Candida albicans*, IC_{50}.
** *Mycobacterium tuberculosis*, MIC.
[††] *Plasmodium falciparum* (malarial parasite), EC_{50}.
[‡‡] COX-1 and COX-2, all extract or compounds non-inhibitory at maximum test concentration of 10 μg/ml.

The first fraction (F3.1) (1.3961 g) was subjected to a flash column chromatography to give three fractions (F3.1.1–F3.1.3). The fraction 3.1.2 (1.1792 g) was further subjected to a flash column to give another three fractions (F3.1.2.1–F3.1.2.3). The fraction 3.1.2.3 (0.468 g) was further purified by PTLC (20% EtOAc/CH_2Cl_2) to give eight fractions (F3.1.2.3.1–F3.1.2.3.8). The fraction 3.1.2.3.6 (0.0467 g) was further purified by PTLC (20% EtOAc/CH_2Cl_2) to give **1** (0.0152 g). The fraction 3.1.2.3.4 (0.0948 g) was purified by PTLC (30% EtOAc/CH_2Cl_2) to give three fractions (F3.1.2.3.4.1–F3.1.2.3.4.3). The fraction 3.1.2.3.4.2 (0.0279 g) was further purified by PTLC twice using 30% EtOAc/CH_2Cl_2 to give **2** (F3.1.2.3.4.2.2.3) (0.0125 g) as a pale yellow powder.

The fraction 3.2 (1.3147 g) was further purified by preparative PTLC (CH_2Cl_2) to give nine fractions (F3.2.1–F3.2.9). The fraction 3.2.2 (0.5862 g) was purified by preparative PTLC (40% EtOAc/hexane) to give six fractions (F3.2.2.1–F3.2.2.6). The fraction 3.2.2.5 (0.2051 g) was further purified by preparative PTLC five times (using 50% EtOAc/hexane, CH_2Cl_2/EtOAc/hexane (5:3:2), 25% EtOAc/CH_2Cl_2, 30% EtOAc/CH_2Cl_2 and 50% EtOAc/hexane as mobile phase, respectively, to give fucoxanthin (**3**) (0.0145 g).

The fraction 3.3 (0.1873 g) was purified by preparative PTLC (50% EtOAc/CH_2Cl_2) to give six fractions (F3.3.1–F3.3.6). The fraction 3.3.3 (0.1254 g) was purified by preparative PTLC (40% EtOAc/CH_2Cl_2) to give another six fractions (F3.3.3.1–F3.3.3.6). The fraction 3.3.3.4 was further purified by preparative PTLC twice with 30% EtOAc/hexane and 50% EtOAc/hexane, respectively to give **4** (0.0042 g).

3.3.1 (1E,2*R,3*R**,4*S**,6*E*,18*S**)-4,18-dihydroxydictyolactone (1)**. A yellow oil. $[\alpha]_D$ −9 (*c* 0.50, CH_2Cl_2). UV λ_{max} nm (CH_2Cl_2) 236 (ε 5163). IR ν_{max} cm^{-1}: 3440, 3045, 2923, 1739, 1644, 1451, 1376. HRESI-MS *m/z* 335.2211 $[M + H]^+$ (calcd for $C_{20}H_{31}O_4$, 335.2222). ^1H and ^{13}C NMR spectral data: see table 1.

3.3.2 8α,11-Dihydroxypachydictyol A (2). A pale yellow powder. $[\alpha]_D$ + 11 (*c* 0.98, CH_2Cl_2). UV λ_{max} nm (CH_2Cl_2) 228 (ε 6756), 408 (ε 5699). IR ν_{max} cm^{-1}: 3479, 3352, 3044, 2960, 1450, 1410, 1385. HRESI-MS *m/z* 343.2249 $[M + Na]^+$ (calcd for $C_{20}H_{32}NaO_3$, 343.2250). ^1H and ^{13}C NMR spectral data: see table 2.

3.3.3 Fucoxanthin (3). A red-coloured oil. ^{1}H NMR and ^{13}C NMR spectral data were consistent with the literature [10].

3.3.4 4α-hydroxycrenulatane (4). A pale oil. ^{1}H NMR and ^{13}C NMR spectral data were consistent with the literature [11–13].

3.4 Generation preparation of MTPA esters

Two portions (each 5 mg) of **1** were treated with S-(+)-α- and (R)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl) chloride (0.5 ml) in a few drops of pyridine, and the mixture was left at room temperature overnight in a closed vial. The reaction mixture was extracted with dichloromethane, dried and evaporated. The residue was checked before further purification by TLC and ^{1}H NMR and found the decomposition.

3.5 Testing for biological activity

All the tests were performed by the National Centre for Genetic Engineering and Biotechnology, Thailand.

3.5.1 Tuberculosis. Anti-tuberculosis testing was performed by the alamar blue susceptibility test (MABA) using *Mycobacterium tuberculosis* H37Ra [14]. Rifampicin, kanamycin and isoniazide were used as positive control.

3.5.2 Herpes simplex virus type 1 (HSV-1). Activity was tested against HSV-1 strain ATCC VR 260, using a colorimetric microtitre plate assay. The assay determined host cell growth by measuring cellular protein content as described by Skehan *et al.* [15]. The growth of host cells, Vero cell line ATCC CCL-81 infected with virus and treated with extract, was compared with control cells, infected with virus only. Acyclovir was used as positive control. The extracts or compounds were tested at non-cytotoxic concentrations (inhibition of cell growth $<50\%$).

3.5.3 Cytotoxicity. Activity was evaluated using the Vero monkey kidney cell line, ATCC CCL-81, and measuring cellular protein content to determine cellular growth [15]. Ellipticine was used as positive control.

3.5.4 Anticancer assays. The assays using KB cells (ATCC CCL-17, a calprotectin-negative oral carcinoma cell line) and BC cells (Breast cancer cell line) were determined, in a similar way, by measuring cellular protein content [15]. Elliptine and doxorubicin were used as positive control. The anticancer assay using NCI-H187 cells (National Cancer Institute human small cell lung carcinoma) was determined by an MTT assay [17].

3.5.5 Antifungal activity. Testing was performed against *Candida albicans* ATCC using a microtitre plate MTT reduction assay [16,17]. Amphotericin B was used as a positive control.

3.5.6 Antiplasmodial activity. Testing was performed against cultures of *Plasmodium falciparum* (K1, multi drug resistant strain) [18]. Quantitative assessment of antimalarial activity *in vitro* was determined by microculture radioisotope techniques based upon the methods described by Desjardins *et al.* [19]. The positive control was dihydroartemisinine (DHA).

3.5.7 Anti-inflammatory assay. The assay has been described by Kirtikara *et al.* [20,21]. Briefly, cultured immortalised mouse PGH-1 and PGH-2 null cells, at the concentration of 1×10^5 cells/ml, were treated with serum-free medium containing vehicle or drugs and 20 μM arachidonic acid (AA) or 2 μM calcium ionophore A23187 for 30 min. Culture supernatants were then collected from wells and analysed for PGE_2 concentrations by ^3H radioimmunoassay (Amersham, USA) to give a measure of cyclooxygenase activity.

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