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Bactericidal and cyclooxygenase inhibitory diterpenes from *Eremophila sturtii*

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Abstract

Two serrulatane diterpenes, 3,8-dihydroxyserrulatic acid (**1**) and serrulatic acid (**2**), have been isolated from *Eremophila sturtii* through bioassay-guided fractionation. These compounds inhibit the inflammation pathway enzymes cyclooxygenase 1 and 2, and exhibit bactericidal activity against *Staphylococcus aureus*.

Keywords: *Eremophila sturtii*; Myoporaceae; Antibacterial; Cyclooxygenase; Lipoxygenase; Serrulatane diterpene

1. Introduction

Eremophila R. Br. is a genus in the Myoporaceae family and is restricted to Australia (Cribb and Cribb, 1981; Ghisalberti, 1995). *Eremophila* species are known to the Australian Aboriginal people as fuchsia bushes or dogwood, and are important to them as medicines (Low, 1990). These plants have been used by the Aboriginal people for colds, fever, sores, wounds, headaches, scabies, and general malaise (Barr et al., 1988; Latz, 1995; Cribb and Cribb, 1981). Although various biological activities such as antibacterial, antiviral, neurological, cardioactive and anti-inflammatory activities have been reported for extracts of *Eremophila* species (Palombo and Semple, 2001, 2002; Semple et al., 1998; Pennacchio et al., 2005; Rogers et al., 2000, 2001, 2002; Sweeney et al., 2001), only a few bioactive components have been characterised (Shah et al., 2004; Pennacchio et al., 1996).

Eremophila sturtii R. Br. (known as turpentine bush, kerosene bush, or turpentine emu bush) is a sticky shrub typically about 2 m high with narrow and cylindrical leaves and small, hairy, white, pink or mauve flowers (Latz, 1995; Cribb and Cribb, 1981). Decoctions of *E. sturtii* leaves have been used by the Australian Aboriginal people of the Northern Territory to wash sores and cuts (Smith, 1991). Through interviews with Murawari elders in northern New South Wales, the authors recorded a similar use of decoctions of crushed leaves of *E. sturtii* 'as a disinfectant' to treat skin infections. These medicinal uses by the Northern Territory and New South Wales Aboriginal communities suggested that *E. sturtii* possesses antimicrobial and/or anti-inflammatory properties. Antibacterial activity against the Gram positive bacterium *Bacillus cereus* has been reported for ethanol extracts of the leaves of *E. sturtii* at a concentration of 1 mg/ml (Palombo and Semple, 2001), but none of its bioactive compounds have been described. In this paper we report the bioassay-guided isolation and structure elucidation of two novel bioactive serrulatic acids (**1** and **2**) from the leaves of *E. sturtii*.

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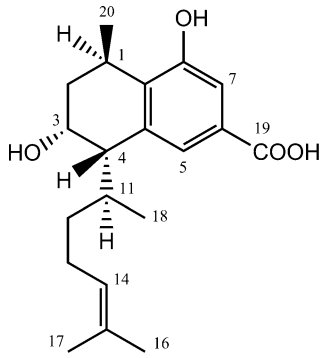

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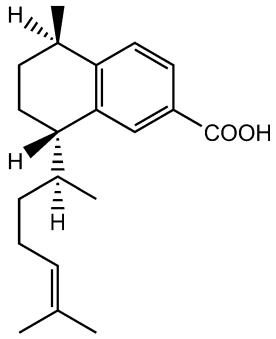

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2. Results and discussion

2.1. Antimicrobial and anti-inflammatory activities of crude extract and fractions

In order to investigate the biological activities of *E. sturtii*, the ethanol crude extract of fresh leaves was examined against a Gram positive bacterium *Staphylococcus aureus*, two Gram negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*, and an yeast *Candida albicans* for its antimicrobial activity, and against the inflammation pathway enzymes cyclooxygenase 1 (COX-1), COX-2 and 5-lipoxygenase (5-LO) for its anti-inflammatory activity. The ethanol extract exhibited antibacterial activity against *S. aureus* with a minimum inhibitory concentration (MIC) of 1.0 mg/ml, but was ineffective against *E. coli*, *P. aeruginosa* and *C. albicans* with concentrations up to 10 mg/ml. The ethanol extract inhibited COX-1 and COX-2 by 95% and 89%, respectively, at 2 mg/ml. No inhibition of 5-LO was observed at 2 mg/ml. The hexane, ethyl acetate, *n*-butanol and water partition fractions of this crude extract were also examined for their antimicrobial and anti-inflammatory activities, and the ethyl acetate partition was found to be the most active, exhibiting an MIC against *S. aureus* of 0.25 mg/ml, and 88% and 66% inhibition of COX-1 and COX-2, respectively, at 2 mg/ml.

2.2. Characterisation of bioactive compounds

Bioassay-guided fractionation of the ethyl acetate partition resulted in the isolation of two novel bioactive compounds, 3,8-dihydroxyserrulatic acid (**1**) and serrulatic acid (**2**), and two known compounds, β-sitosterol and sesamin. Their structures were elucidated by 1D and 2D NMR spectroscopy and mass spectrometry. β-Sitosterol and sesamin were identified by comparing their NMR and mass spectral data with reported data (Kovganko et al., 2000; Jayasinghe et al., 2003).

Compound **1** was isolated as an amorphous white solid, having a molecular formula of $C_{20}H_{28}O_4$ (*m*/*z* 332.1976, calcd. 332.1988) in the HREIMS. The ^{13}C and DEPT NMR spectra indicated a total of 20 carbons (Table 1), with 4 being methyls (δ_C 18.4, 19.7, 22.9 and 26.4), 3 methylenes (δ_C 26.7, 35.5 and 39.7), 3 sp^2 methines (δ_C 113.8, 122.5 and 125.7), 4 sp^3 methines (δ_C 29.1, 30.7, 49.7 and 65.6), and 6 quaternary carbons (δ_C 128.8, 131.2, 134.4, 139.8, 155.6 and 168.5). The ^1H NMR spectrum showed three very broad singlets at δ_H 12.54, 9.58 and 4.70 in DMSO-d_6. These peaks disappeared when D_2O was added.

HMBC correlations showed that two aromatic protons in a *meta* relationship (δ_H 7.19 and 7.07, each *d*, $J = 1.5$ Hz) were located on either side of a quaternary carbon (δ_C 128.8) with a carboxylic acid substituent (δ_H 12.54, *br. s*, δ_C 168.5) and that the aromatic proton at 7.19 ppm was next to a quaternary carbon (δ_C 155.6) with a phenolic group (δ_H 9.58, *br. s*). Two benzylic methine groups (δ_H 3.14, *m*, δ_C 29.1 and δ_H 2.77, *br. d*, $J = 4.5$ Hz, δ_C 49.7) were found in the ^1H and ^{13}C NMR spectra, with the latter showing a HMBC correlation with the aromatic proton at 7.07 ppm. The HMBC correlations of the aromatic moiety of compound **1** are shown in Fig. 1. The benzylic methine proton at 3.14 ppm, which flanked with a secondary methyl group (δ_H 1.16, *d*, $J = 6.9$ Hz, δ_C 22.9), showed couplings with two protons on a methylene carbon (δ_H 1.88, *ddd*, $J = 12$, ~6.5, ~6.5 Hz and δ_H 1.46, *m*; δ_C 35.5) in the COSY spectrum. A hydroxyl bearing methine (δ_H 4.07,

Table 1
^1H and ^{13}C NMR data for compounds **1** and **2**

C/H	Compound **1**		Compound **2**	
	$\delta^{13}C$	δ^1H (*J*/Hz)	$\delta^{13}C$	δ^1H (*J*/Hz)
1	29.1	3.14, *m*	33.7	2.77, *m*
2	35.5	1.88 (2a), *ddd* (12, ~6.5, ~6.5)	31.2	1.90, *m*
		1.46 (2b), *m*		1.28, *m*
3	65.6	4.07, *ddd* (12, 4.5, ~4)	22.5	1.82, *m*
				1.48, *m*
4	49.7	2.77, *br. d* (4.5)	43.9	2.77, *m*
5	122.5	7.07, *d* (1.5)	129.7	7.78, *d* (1.7)
6	128.8		128.9	
7	113.8	7.19, *d* (1.5)	127.1	7.65, *dd* (8.1, 1.7)
8	155.6		128.0	7.31, *d* (8.1)
9	134.4		148.7	
10	139.8		140.4	
11	30.7	2.13, *m*	36.7	2.04, *m*
12	39.7	1.48, *m*	32.1	0.99, *m*
		1.26, *m*		
13	26.7	1.97, *m*	26.5	1.81, *m*
14	125.7	5.12, *tqq* (7.1, 1.3, 0.8)	125.2	4.89, *tqq* (7.0, 1.3, 0.9)
15	131.2		131.7	
16	26.4	1.63, *d* (0.8)	26.3	1.56, *d* (0.9)
17	18.4	1.55, *d* (1.3)	18.3	1.44, *d* (1.3)
18	19.7	0.33, *d* (7.0)	18.7	0.95, *d* (6.7)
19	168.5		168.5	
20	22.9	1.16, *d* (6.9)	22.8	1.21, *d* (6.8)
	–OH	4.70, *br. s*		
	–OH	9.58, *br. s*		
	–COOH	12.54, *br. s*		

Spectra obtained at 400 MHz (^{13}C at 100 MHz) in DMSO-d_6.

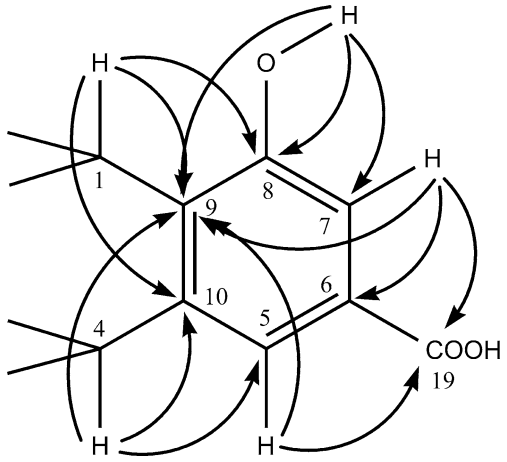

Fig. 1. HMBC correlations of the aromatic moiety of compound **1**.

ddd, $J = 12$, 4.5, ~4 Hz, δ_{-OH} 4.70, δ_C 65.6) connected the methylene and the benzylic methine group at 2.77 ppm (C-4) to form a bicyclic moiety.

The remaining signals in the ^1H and ^{13}C NMR spectra were assigned as a side chain connected to the bicyclic moiety at C-4. These signals consisted of a trisubstituted vinyl group (δ_H 5.12, *tqq*, $J = 7.1$, 1.3, 0.8 Hz, δ_C 125.7 and 131.2), two methylenes (δ_H 1.97, 2H, *m*, δ_C 26.7 and δ_H 1.48, *m*, 1.26, *m*, δ_C 39.7), an alkyl methine (δ_H 2.13, *m*, δ_C 30.7), and three methyl groups (δ_H 1.55, *d*, $J = 1.3$ Hz, δ_C 18.4, δ_H 1.63, *d*, $J = 0.8$ Hz, δ_C 26.4 and δ_H 0.33, *d*, $J = 7.0$ Hz, δ_C 19.7). The COSY spectrum confirmed the couplings of the olefinic proton with the methylene protons at 1.97 ppm and vinylic methyl protons at 1.55 and 1.63 ppm, and the couplings of the methine proton to the methyl protons at 0.33 ppm and the methylene protons at 1.48 ppm. Attachment of the methine group to the C-4 of the bicyclic system was determined by its HMBC correlations with the hydroxymethine carbon (δ_C 65.6) and a quaternary aromatic carbon (δ_C 139.8).

The spectral data were in agreement with compound **1** being a diterpene with a serrulatane skeleton. This compound is a positional isomer of the previously reported dihydroxyserrulatic acid isolated from *Eeremophila serrulate* (Croft et al., 1979; Foster et al., 1986). To follow the general name used in the previous paper, compound **1** was given the trivial name 3,8-dihydroxyserrulatic acid. The assignment of the NMR data of compound **1** is shown in Table 1.

The relative stereochemistry of compound **1** at C-1, C-3 and C-4 was determined by 2D ROESY NMR experiments and by comparing the coupling constants with published data. In the ROESY spectrum, strong correlations were found between H-3 and H-20, H-3 and H-4, and H-4 and H-5. The coupling constants of $J_{3,4}$ 4.5, $J_{2b,3} \sim 4$ and $J_{2a,3}$ 12 Hz were consistent with the cyclohexene ring adopting a distorted chair conformation with the methyl group (H-20) and H-3 both pseudoaxial and the 3-OH group and H-4 pseudoequatorial (Syah and Ghisalberti, 1997; Tippett and Massy-Westropp, 1993). Furthermore, the significant upfield shift of the H-18 methyl resonance (δ_H 0.33) was in agreement with other serrulatane diterpenes with a secondary hydroxyl group pseudoequatorial at C-3 (Syah and Ghisalberti, 1997; Tippett and Massy-Westropp, 1993).

Compound **2** was isolated as an amorphous white solid, having a molecular formula of $C_{20}H_{28}O_2$ (*m*/*z* 300.2090, calcd. 300.2089) in the HREIMS, i.e. two oxygen atoms less than compound **1**. The ^{13}C and DEPT NMR identified 20 carbons, with 4 being methyls (δ_C 18.3, 18.7, 22.8 and 26.3), 4 methylenes (δ_C 22.5, 26.5, 31.2 and 32.1), 4 sp^2 methines (δ_C 125.2, 127.1, 128.0 and 129.7), 3 sp^3 methines (δ_C 33.7, 36.7 and 43.9), and 5 quaternary carbons (δ_C 128.9, 131.7, 140.4, 148.7 and 168.5). Compounds **1** and **2** showed similar ^1H NMR spectra, with the main difference being the absence of the phenolic proton (δ_H 9.58) and the hydroxymethyl group (δ_H 4.07 and 4.70) signals for compound **2**. Three aromatic protons were found for compound **2** (δ_H 7.78, *d*, $J = 1.7$ Hz; 7.65, *dd*, $J = 8.1$, 1.7 Hz and 7.31, *d*, $J = 8.1$ Hz), consistent with a 1, 2, 4 relationship. HMBC correlations confirmed the presence of a carboxylic group (δ_C 168.5) located at C-6, between the two aromatic protons at 7.78 and 7.65 ppm. Two benzylic protons overlapped with each other and gave a multiplet at 2.77 ppm. The bicyclic partial structure of compound **2** was confirmed by the coupling of the benzylic protons with two joined methylene groups (δ_H 1.90, *m*, and 1.28, *m*, δ_C 31.2; and δ_H 1.82, *m*, and 1.48, *m*, δ_C 22.5) in the COSY spectrum.

The assignment of the side chain for compound **2** was very similar to that of compound **1**, with the only significant difference being the methyl group at position 18 resonated at 0.95 ppm. This chemical shift is consistent with other serrulatane diterpenes that lack a secondary hydroxyl group at C-3 (Foster et al., 1986; Croft et al., 1979, 1981; Ghisalberti et al., 1990). The assignments of all ^1H and ^{13}C NMR data for compound **2** are shown in Table 1.

The relative stereochemistry of compound **2** was unable to be determined by nOe NMR experiments as both of the benzylic protons (H-1 and H-4) had the same chemical shift and their correlations with vicinal protons were indistinguishable. Due to its similarity to compound **1** and other serrulatane diterpenes, the stereochemistry of compound **2** was assumed to be the same as that of compound **1**. Compound **2** is referred to as serrulatic acid to be consistent with the trivial name used by Croft et al. (1979).

2.3. Biological activities of novel compounds

The two novel compounds (**1** and **2**) were examined for their antimicrobial and anti-inflammatory activities (Table 2). Compound **1** exhibited bactericidal activity against *S. aureus* with the minimum bactericidal concentration (MBC) of 200 μg/ml. No activity was observed against *E. coli*, *P. aeruginosa* or *C. albicans*. Compound **1** had weak COX-1 and COX-2 inhibitory activity with 57%

Table 2
Inhibitory activities against inflammation pathway enzymes and bactericidal activities of compound **1** and **2**

Compound	% Inhibition at 1 mg/ml			Minimum bactericidal concentration (μg/ml)			
	COX-1	COX-2	5-LO	*S. aureus*	*E. coli*	*P. aeruginosa*	*C. albicans*
1	57	14	–	200	>1000	>1000	>1000
2	99	97	–	15	>400	>400	>400

and 14% inhibition, respectively, at 1 mg/ml. Compound **2** demonstrated potent bactericidal activity against *S. aureus* with a MBC of 15 μg/ml. It strongly inhibited both COX-1 and COX-2 at 1 mg/ml with 99% and 97% inhibition, respectively. IC_{50} values of 27 μg/ml for COX-1 and 73 μg/ml for COX-2 were also obtained for compound **2**. The positive control ibuprofen exhibited IC_{50} values of 0.1 and 0.6 μg/ml for COX-1 and COX-2, respectively. No inhibition of 5-LO was observed with either compound **1** or compound **2**.

3. Experimental

3.1. General

Merck silica gel 60, Fluka reversed phase C_{18} silica gel 100 and Supelco MCI gel CHP20P were used for column chromatography. High performance liquid chromatography (HPLC) experiments were performed on Shimadzu LC-10A and LC-10A *vp* systems with Waters Sunfire analytical (4.6 mm × 150 mm) and semi-preparative (10 mm × 150 mm) HPLC columns. 1D and 2D NMR spectra were acquired on a Bruker DPX400 spectrometer. IR spectra were measured on a Perkin–Elmer Paragon 1000 PC FT-IR spectrometer. UV spectra were recorded on a Varian Cary 1 BIO UV–visible spectrophotometer. Melting points (uncorrected) were determined by a Stuart Scientific SMP10 apparatus. Optical rotations were measured on a Jasco P-1010 polarimeter. High resolution mass spectra (HREIMS) were obtained on a VG AutoSpec M series sector (EBE) mass spectrometer, and low resolution mass spectra were acquired on a Shimadzu GCMS-QP5000 gas chromatograph mass spectrometer.

3.2. Plant material

Plant sample (fresh leaves) of *E. sturtii* was collected from Lightning Ridge, New South Wales in March 2004. A voucher specimen was deposited in the herbarium of Macquarie University (Voucher No. 73007710).

3.3. Extraction and isolation of metabolites from E. sturtii

Fresh leaves of *E. sturtii* (648 g) were blended with 70% (v/v) ethanol and water (1.6 L) and stirred occasionally at room temperature for 1 day before being filtered. The extraction was repeated twice with increasing stirring time (up to 3 days) and the filtrates were combined and evaporated under reduced pressure to give 94 g of dark green crude extract. The crude extract was suspended in 250 ml water and partitioned with hexane (3 × 150 ml), ethyl acetate (3 × 150 ml) and *n*-butanol (4 × 100 ml) sequentially to give four fractions. The ethyl acetate fraction (22.6 g, dark green) was found to be the most active in the biological assays.

The ethyl acetate fraction was subjected to flash silica gel column chromatography with petroleum ether and acetone (100:1 to 1:100, v/v). 11 fractions were collected based on their TLC pattern and labelled as Es-EtOAc-1 to Es-EtOAc-11 in order of increasing polarity. Fraction Es-EtOAc-2 (190 mg) was washed with methanol until colourless and recrystallised in acetone to afford β-sitosterol (19 mg) as needles, m.p. 132–134 °C (lit. 135 °C) (Pacheco et al., 1973). ^1H and ^{13}C NMR data were in agreement with those reported in the literature (Kovganko et al., 2000). Fraction Es-EtOAc-3 (1.7 g) was purified by normal and reversed phase silica gel column chromatography to give after recrystallisation with methanol sesamin (27 mg) as colourless crystals, m.p. 122–123 °C (lit. 124 °C) (Jayasinghe et al., 2003). ^1H and ^{13}C NMR and mass spectrometric data were identical to those published in the literature (Jayasinghe et al., 2003).

Fraction Es-EtOAc-5 (9.1 g, eluted with petroleum ether and acetone 5:1, v/v) was subjected to flash silica gel column chromatography with petroleum ether and ethyl acetate (7:1 to 1:3, v/v) and 8 fractions Es-EtOAc-5-1 to Es-EtOAc-5-8 were collected in order of increasing polarity. All of these fractions showed antibacterial activity against *S. aureus*, with Es-EtOAc-5-3 to Es-EtOAc-5-5 (eluted with petroleum ether and ethyl acetate 2:1 v/v) being the most active fractions. Fraction Es-EtOAc-5-5 (2.1 g) was separated by reversed phase MCI gel column chromatography using methanol and water 55:45 to 90:10 (v/v) and 9 fractions Es-EtOAc-5-5-1 to Es-EtOAc-5-5-9 were collected in order of elution. Es-EtOAc-5-5-3 (488 mg, eluted with methanol and water 80:20, v/v) was further separated by reversed phase RP-18 column chromatography using methanol and water 55:45 to 75:25 (v/v). The major component Es-EtOAc-5-5-3-2 (collected with methanol and water 65:35, v/v) was then dissolved in methanol and crystallised by adding water until a white solid just appeared. After overnight storage at 4 °C, 285 mg of compound **1** was obtained as a white solid.

Fraction Es-EtOAc-1 (3.1 g, eluted with petroleum ether and acetone 100:1 to 20:1, v/v) was subjected to flash silica gel column chromatography with petroleum ether and ethyl acetate (15:1 to 1:5, v/v). 15 fractions Es-EtOAc-1-1 to Es-EtOAc-1-15 were collected in order of polarity.

Fraction Es-EtOAc-1-8 (0.5 g, collected with petroleum ether and acetone 10:1, v/v) was then separated by a reversed phase RP-18 silica gel column chromatography with methanol and water (60:40 to 97:3, v/v) and 10 fractions Es-EtOAc-1-8-1 to Es-EtOAc-1-8-10 were collected in order of elution. Es-EtOAc-1-8-4 (collected with methanol and water 95:5, v/v) was further cleaned by semi-preparative HPLC repeatedly (acetonitrile:water 88:12, v/v, 4 ml/min) to give 191 mg of pure compound **2** as a white solid.

*3.3.1. 3,8-Dihydroxyserrulatic acid (**1**)*

M.p. 173–174 °C; $[\alpha]_D^{23} + 14.1^\circ$ (methanol, *c* 0.155); UV λ_{max}^{MeOH} nm ($\log \varepsilon$): 293 (3.2), 242 (3.8), 212 (4.5); IR (KBr) ν_{max} cm^{-1}: 2968, 2928, 1685, 1588, 1424, 1236, 1056, 702; HREIMS *m/z*: 332.1976 $[M]^+$, LREIMS *m/z*: 332, 314, 247, 232, 217, 204, 189, 177. ^1H and ^{13}C NMR data are shown in Table 1.

*3.3.2. Serrulatic acid (**2**)*

M.p. 82–83 °C; $[\alpha]_D^{22} - 32.2^\circ$ (methanol, *c* 0.102); HREIMS *m/z*: 300.2090 $[M]^+$, LREIMS *m/z*: 300, 255, 244, 216, 189, 145, 129, 117, 115, 105, 91. ^1H and ^{13}C NMR data are shown in Table 1.

3.4. Antimicrobial assays

3.4.1. Microorganisms

Gram positive bacterium *S. aureus* ATCC 9144, Gram negative *E. coli* JM109 and *P. aeruginosa* ATCC 27853, and an yeast *C. albicans* AMMRL 36.42, were used in the antimicrobial assays. Single colonies of *S. aureus*, *E. coli* and *P. aeruginosa* from Mueller-Hinton agar (Becton Dickinson) and of *C. albicans* from Sabouraud dextrose agar (Oxoid) were inoculated into Mueller-Hinton broth (for *S. aureus*, *E. coli* and *P. aeruginosa*) or Sabouraud dextrose broth (for *C. albicans*) and incubated overnight at 37 °C. The optical density of each culture was adjusted to 0.08 at 600 nm and a 1/100 dilution of this was used in the antimicrobial assays.

3.4.2. Fluorescein diacetate (FDA) assay

The FDA assay (Wanandy et al., 2005) was performed in black flat-bottom 96-well microtitre plates (Greiner) to determine the MIC of the crude extract, fractions and pure compounds against *P. aeruginosa*, *E. coli* and *S. aureus*. In this assay, 175 μl of inoculum and 20 μl of serial dilutions of sample were added into a microtitre plate and incubated overnight (16–18 h) at 37 °C. Geneticin (Invitrogen) and tetracycline (Sigma) were used as the positive controls and 175 μl of liquid media and 20 μl of sample were used as the background control. Samples and controls were assayed in triplicates. 5 μl of 2 mg/ml FDA solution (in acetone) was added into each well followed by an additional 3 h incubation. Fluorescence intensity values were recorded by a BMG Fluostar Galaxy microtitre plate reader. The lowest concentration of sample at which no increase of fluorescence intensity was observed was recorded as the minimum inhibitory concentration (MIC). To determine whether the activity was bacteriostatic or bactericidal, samples from microwells showing inhibition were plated and viable counts were compared with those from control microwells.

3.4.3. Disc diffusion assay

The disc diffusion method (National Committee for Clinical Laboratory Standards, 2001) was used to test the plant extracts and compounds for activity against *C. albicans* on Sabouraud dextrose agar plates using 6 mm diameter filter paper discs.

3.5. Cyclooxygenase and lipoxygenase inhibitor screening assays

Cyclooxygenase and lipoxygenase inhibitor screening assay kits (Cayman Chemicals) were used to evaluate COX-1, COX-2 and 5-LO inhibitory activities of the plant extracts and compounds. Ovine COX-1, human recombinant COX-2 and potato 5-LO (Cayman Chemicals) were used in these assays. Experimental procedures were performed according to the manufacturer's instructions. Ibuprofen was used as a control in the COX assay.

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