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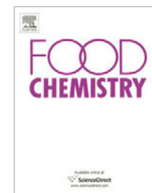

Sesquiterpenes from the rhizome of *Curcuma longa* with inhibitory activity on superoxide generation and elastase release by neutrophils

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ABSTRACT

Six new sesquiterpenes, curculonone A (**1**), curculonone B (**2**), curculonone C (**3**), curculonone D (**4**), 6α-hydroxycurcumanolide A (**5**), and 1,10-dehydro-10-deoxy-9-oxozedoarondiol (**6**), have been isolated from the rhizome of *Curcuma longa*, together with 19 known compounds. The structures of these new compounds were determined through spectroscopic and MS analyses. Compounds **1**, **2**, **5**, **12**, **15**, **16**, and **23** exhibited inhibition ($IC_{50} \leqslant 18.22$ μM) of superoxide anion generation by human neutrophils in response to formyl-L-methionyl-L-leucyl-L-phenylalanine/cytochalasin B (fMLP/CB). Compounds **5**, **12–16**, and **23** inhibited fMLP/CB-induced elastase release with IC_{50} values $\leqslant$ 14.28 μM.

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1. Introduction

Curcuma longa L. (Zingiberaceae) (Kan, 1997) is a perennial herb, distributed throughout tropical and subtropical regions of the world, being widely cultivated in Asiatic countries, mainly in India and China. The rhizomes of *C. longa* have been widely used as a yellow colouring agent and spice in many foods (Kan, 1997). Sesquiterpenoids (Itokawa, Hirayama, Funakoshi, & Takeya, 1985; Kiso, Suzuki, Oshima, & Hikino, 1983; Kouno & Kawano, 1985; Ohshiro, Kuroyanagi, & Ueno, 1990; Shiobara, Asakawa, Kodama, Yasuda, & Takemoto, 1985), curcuminoids (Masuda, Jitoe, Isobe, Nakatani, & Yonemori, 1993; Park et al., 2005), curcumin-related phenolics (Masuda et al., 1993), and their derivatives are widely distributed in plants of the genus *Curcuma*. Many of these compounds exhibit antitumor (Itokawa et al., 1985), anti-oxidative (Masuda et al., 1993), anti-inflammatory (Hong et al., 2004; Masuda et al., 1993), and hypoglycaemic (Nishiyama et al., 2005) activities. In our studies on the anti-inflammatory constituents of Formosan plants, many species have been screened for *in vitro* inhibitory activity on neutrophil pro-inflammatory responses, and *C. longa* has been found to be an active species. Six new sesquiterpenes, curculonone A (**1**), curculonone B (**2**), curculonone C (**3**), curculonone D (**4**), 6α-hydroxycurcumanolide A (**5**), and 1,10-dehydro-10-deoxy-9-oxozedoarondiol (**6**) and 19 known compounds have been isolated and identified from the rhizome of *C. longa*. This paper describes the structural elucidation of **1**–**6** and their inhibitory activity on superoxide generation and elastase release by neutrophils.

2. Materials and methods

2.1. General

Optical rotations were measured using a Jasco DIP-370 polarimeter in $CHCl_3$. CD spectra were recorded on a Jasco J-810 spectropolarimeter. UV spectra were obtained on a Jasco UV-240 spectrophotometer. IR spectra (neat or KBr) were recorded on a Perkin Elmer 2000 FT-IR spectrometer. NMR spectra, including COSY, NOESY, HMBC, and HSQC experiments, were recorded on a Varian Unity 400 or a Varian Inova 500 spectrometer operating at 400 and 500 MHz (^1H) and 100 and 125 MHz (^{13}C), respectively, with chemical shifts given in ppm (δ) using TMS as an internal standard. ESI and HR-ESI-mass spectra were recorded on a Bruker

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APEX II mass spectrometer. Silica gel (70–230, 230–400 mesh) (Merck) was used for CC. Silica gel 60 F-254 (Merck) was used for TLC and PTLC.

2.2. Plant material

The rhizome of *C. longa* was collected from Taitung District Agricultural Research and Extension Station, Taitung County, Taiwan, in April 2007 and identified by J.-F. Chen. A voucher specimen (118962) was deposited in the Herbarium of Taiwan Forestry Research Institute, Taipei, Taiwan.

2.3. Extraction and isolation

The dried rhizome of *C. longa* (5.4 kg) was pulverised and extracted 3× with MeOH (20 l each) for 3 days. The MeOH extracts were concentrated under reduced pressure at 35 °C, and the residue (650 g) was partitioned between CH_2Cl_2 and H_2O (1:1). The CH_2Cl_2 layer was concentrated to give a residue (fraction A, 120 g). The water layer was further extracted with *n*-BuOH, and the *n*-BuOH-soluble part (fraction B, 225 g) and the water-solubles (fraction C, 295 g) were separated. Fraction A (120 g) was chromatographed on silica gel (70–230 mesh, 4.5 kg), eluting with CH_2Cl_2, gradually increasing the polarity with MeOH to give 14 fractions: A1 (6 l, CH_2Cl_2), A2 (7 l, CH_2Cl_2/MeOH, 95:1), A3 (4 l, CH_2Cl_2/MeOH, 90:1), A4 (3 l, CH_2Cl_2/MeOH, 85:1), A5 (3 l, CH_2Cl_2/MeOH, 80:1), A6 (4 l, CH_2Cl_2/MeOH, 70:1), A7 (4 l, CH_2Cl_2/MeOH, 50:1), A8 (6 l, CH_2Cl_2/MeOH, 30:1), A9 (5 l, CH_2Cl_2/MeOH, 10:1), A10 (3 l, CH_2Cl_2/MeOH, 5:1), A11 (5 l, CH_2Cl_2/MeOH, 3:1), A12 (7 l, CH_2Cl_2/MeOH, 2:1), A13 (3 l, CH_2Cl_2/MeOH, 1:1), and A14 (8 l, MeOH). Fraction A1 (5.8 g) was chromatographed further on silica gel (230–400 mesh, 215 g) eluting with *n*-hexane/acetone (30:1) to give 10 fractions (each 1.5 l, A1-1–A1-10). Fraction A1-2 (178 mg) was purified further by preparative TLC (silica gel, *n*-hexane/acetone, 15:1) to obtain **10** (3.8 mg) (R_f = 0.87). Fraction A1-5 (190 mg) was purified further by preparative TLC (silica gel, *n*-hexane/acetone, 20:1) to yield **9** (3.6 mg) (R_f = 0.63). Fraction A2 (7.8 g) was chromatographed further on silica gel (230–400 mesh, 265 g) eluting with *n*-hexane/acetone (10:1) to give 11 fractions (each 1.0 l, A2-1–A2-11). Fraction A2-3 (220 mg) was purified further by preparative TLC (silica gel, *n*-hexane/CH_2Cl_2, 1:1) to obtain **7** (3.5 mg) (R_f = 0.50). Fraction A3 (8.5 g) was chromatographed on silica gel (230–400 mesh, 235 g) eluting with *n*-hexane/acetone (10:1) to give 14 fractions (each 1.2 l, A3-1–A3-14). Fraction A3-1 (195 mg) was purified further by preparative TLC (silica gel, *n*-hexane/EtOAc, 15:1) to yield **11** (4.4 mg) (R_f = 0.73). Fraction A3-3 (192 mg) was purified further by preparative TLC (silica gel, *n*-hexane/EtOAc, 1:1) to afford **1** (3.3 mg) (R_f = 0.52). Fraction A3-4 (205 mg) was purified further by preparative TLC (silica gel, *n*-hexane/EtOAc, 15:1) to obtain **2** (4.0 mg) (R_f = 0.78). Fraction A3-5 (187 mg) was purified further by preparative TLC (silica gel, *n*-hexane/acetone, 3:1) to obtain **3** (3.7 mg) (R_f = 0.60). Fraction A3-7 (175 mg) was purified further by preparative TLC (silica gel, CH_2Cl_2/acetone, 20:1) to afford **18** (5.2 mg) (R_f = 0.63). Fraction A3-8 (185 mg) was purified further by preparative TLC (silica gel, CH_2Cl_2/acetone, 15:1) to afford **22** (3.0 mg) (R_f = 0.69). Fraction A4 (8.2 g) was chromatographed on silica gel (230–400 mesh, 285 g) eluting with *n*-hexane/EtOAc (5:1) to give 13 fractions (each 1 l, A4-1–A4-13). Fraction A4-1 (355 mg) was washed with MeOH and filtered to yield a mixture of **24** and **25** (45 mg) after recrystallisation (MeOH). Fraction A4-5 (230 mg) was separated by MPLC (78 g silica gel, 230–400 mesh, *n*-hexane/EtOAc, 10:1, 150 ml fractions) to obtain 8 subfractions: A4-5-1–A4-5-8. Fraction A4-5-3 (32 mg) was purified further by preparative TLC (silica gel, *n*-hexane/acetone, 10:1) to yield **13** (4.1 mg) (R_f = 0.80). Fraction A4-5-4 (28 mg) was purified further by preparative TLC (silica gel, *n*-hexane/acetone, 10:1) to yield **14** (2.9 mg) (R_f = 0.81). Fraction A4-7 (180 mg) was purified further by preparative TLC (silica gel, *n*-hexane/acetone, 8:1) to afford **5** (5.4 mg) (R_f = 0.41). Fraction A5 (7.3 g) was chromatographed on silica gel (230–400 mesh, 255 g) eluting with *n*-hexane/EtOAc (2:1) to give 10 fractions (each 800 ml, A5-1–A5-10). Fraction A5-2 (202 mg) was purified further by preparative TLC (silica gel, $CHCl_3$/acetone, 20:1) to obtain **23** (3.8 mg) (R_f = 0.63). Fraction A5-3 (188 mg) was purified further by preparative TLC (silica gel, $CHCl_3$/acetone, 15:1) to obtain **15** (35 mg) (R_f = 0.68). Fraction A5-4 (210 mg) was purified further by preparative TLC (silica gel, $CHCl_3$/acetone, 15:1) to yield **16** (23 mg) (R_f = 0.64). Fraction A5-5 (205 mg) was purified further by preparative TLC (silica gel, $CHCl_3$/acetone, 15:1) to yield **17** (18 mg) (R_f = 0.52). Fraction A6 (7.7 g) was chromatographed on silica gel (230–400 mesh, 260 g) eluting with $CHCl_3$/acetone (5:1) to give 8 fractions (each 1.6 l, A6-1–A6-8). Fraction A6-3 (185 mg) was purified further by preparative TLC (silica gel, $CHCl_3$/MeOH, 10:1) to obtain **8** (4.2 mg) (R_f = 0.83). Fraction A8 (6.8 g) was chromatographed on silica gel (230–400 mesh, 245 g) eluting with EtOAc to give 10 fractions (each 700 ml, A8-1–A8-10). Fraction A8-3 (200 mg) was purified further by preparative TLC (silica gel, CH_2Cl_2/acetone, 5:1) to obtain **20** (3.0 mg) (R_f = 0.72). Fraction A8-4 (180 mg) was purified further by preparative TLC (silica gel, CH_2Cl_2/acetone, 5:1) to afford **21** (2.8 mg) (R_f = 0.71). Fraction A8-6 (210 mg) was purified further by preparative TLC (silica gel, CH_2Cl_2/acetone, 10:1) to obtain **6** (3.4 mg) (R_f = 0.60) and **19** (3.2 mg) (R_f = 0.63). Fraction A8-7 (195 mg) was purified further by preparative TLC (silica gel, $CHCl_3$/MeOH, 8:1) to obtain **12** (3.9 mg) (R_f = 0.57). Fraction A8-10 (175 mg) was purified further by preparative TLC (silica gel, $CHCl_3$/MeOH, 6:1) to obtain **4** (2.8 mg) (R_f = 0.13).

Curculonone A (**1**): colourless oil; $[\alpha]_D^{25}$ − 38.4 (*c* 0.13, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 235 (4.07) nm; CD (MeOH) $[\theta]_{393}$ − 285, $[\theta]_{365}$ 0, $[\theta]_{345}$ + 397, $[\theta]_{305}$ 0, $[\theta]_{264}$ − 1280, $[\theta]_{251}$ − 3475, $[\theta]_{241}$ − 5655; IR (neat) ν_{max} 3435 (OH), 1670 (C=O) cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR ($CDCl_3$, 100 MHz): δ 15.6 (C-14), 24.1 (C-15), 26.0 (C-6), 29.9 (C-12), 29.9 (C-13), 30.5 (C-5), 33.2 (C-7), 37.0 (C-8), 49.8 (C-1), 70.3 (C-11), 125.6 (C-9), 127.0 (C-3), 139.7 (C-10), 161.2 (C-4), 201.0 (C-2); ESIMS *m/z* 259 $[M+Na]^+$; HRESIMS *m/z* 259.1676 $[M+Na]^+$ (calcd for $C_{15}H_{24}O_2Na$, 259.1674).

Curculonone B (**2**): colourless oil; $[\alpha]_D^{25}$ + 58.7 (*c* 0.11, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 234 (4.02) nm; CD (MeOH) $[\theta]_{392}$ + 368, $[\theta]_{362}$ 0, $[\theta]_{343}$ − 328, $[\theta]_{315}$ 0, $[\theta]_{270}$ + 2235, $[\theta]_{251}$ + 5660, $[\theta]_{240}$ + 4152; IR (neat) ν_{max} 3440 (OH), 1670 (C=O) cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR ($CDCl_3$, 100 MHz): δ 15.6 (C-14), 24.1 (C-15), 26.1 (C-6), 29.9 (C-12), 29.9 (C-13), 30.6 (C-5), 33.3 (C-7), 37.2 (C-8), 49.9 (C-1), 70.3 (C-11), 125.7 (C-9), 127.0 (C-3), 139.5 (C-10), 161.1 (C-4), 201.0 (C-2); ESIMS *m/z* 259 $[M+Na]^+$; HRESIMS *m/z* 259.1673 $[M+Na]^+$ (calcd for $C_{15}H_{24}O_2Na$, 259.1674).

Curculonone C (**3**): colourless oil; $[\alpha]_D^{25}$ − 44.6 (*c* 0.12, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 234 (4.03) nm; CD (MeOH) $[\theta]_{386}$ − 261, $[\theta]_{367}$ 0, $[\theta]_{340}$ + 352, $[\theta]_{303}$ 0, $[\theta]_{263}$ − 1422, $[\theta]_{245}$ − 4368; IR (neat) ν_{max} 1672 (C=O) cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR ($CDCl_3$, 100 MHz): δ 15.6 (C-14), 18.7 (C-13), 24.1 (C-15), 24.9 (C-12), 25.9 (C-9), 26.0 (C-6), 30.4 (C-5), 30.7 (C-7), 32.8 (C-8), 49.9 (C-1), 58.3 (C-11), 64.8 (C-10), 127.0 (C-3), 161.1 (C-4), 201.0 (C-2); ESIMS *m/z* 259 $[M+Na]^+$; HRESIMS *m/z* 259.1674 $[M+Na]^+$ (calcd for $C_{15}H_{24}O_2Na$, 259.1674).

Curculonone D (**4**): colourless oil; $[\alpha]_D^{25}$ − 30.5 (*c* 0.12, $CHCl_3$); CD (MeOH) $[\theta]_{320}$ + 518; IR (neat) ν_{max} 3390 (OH) cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ 0.88 (3H, d, *J* = 6.8 Hz, H-14), 1.31 (3H, s, H-12), 1.31 (3H, s, H-13), 1.31 (3H, s, H-15), 1.72 (1H, m, H-7), 1.73 (1H, dt, *J* = 14.0, 7.0 Hz, H_α-6), 1.83 (1H, ddd, *J* = 14.0, 7.2, 3.2 Hz, H_β-6), 2.01 (2H, br t, *J* = 6.4 Hz, H-8), 2.31 (1H, m, H-1), 3.79 (1H, dd, *J* = 7.0, 3.2 Hz, H-5), 5.60 (1H, dt, *J* = 15.6, 6.4 Hz, H-9), 5.65 (1H, s, H-2), 5.65 (1H, s, H-3), 5.65 (1H, d, *J* = 15.6 Hz, H-10); ^{13}C

Table 1
^{1}H NMR data of **1–3**.[a]

Position	1	2			3		
	δ_H *J* (Hz)	δ_H *J* (Hz)	NOE	HMBC	δ_H *J* (Hz)	NOE	HMBC
1	2.17 ddd (12.0, 4.0, 4.0)	2.16 m	6, 14	2, 3, 14	2.16 br d (12.4)	6, 7	2, 3, 5
3	5.86 s	5.86 s	15	1, 2, 15	5.87 s	15	1, 2, 5
5	2.27–2.32 m	2.27–2.32 m	6, 15	1, 3	2.28–2.34 m	6, 15	1, 3, 15
6	1.77 m	1.78 m	5	2, 4	1.80 m	1, 5	2, 7
	1.92 m	1.94 m	1, 5	2, 4, 7	1.94 m	5	2, 4, 7
7	2.40 m	2.39 m	8, 14	2, 6, 9	2.35 m	1, 8	2, 6, 9
8	2.00 t (6.4)	2.05 t (6.8)	7, 9, 10	1, 10, 14	1.38 m	7, 9	1, 10
9	5.58 dt (15.6, 6.4)	5.65 dt (15.6, 6.8)	8, 13	7, 11	1.50 m	8, 10	7, 11
10	5.64 d (15.6)	5.56 d (15.6)	8, 12	8, 11, 12	2.71 br t (6.2)	9, 13	8, 11, 12
12	1.30 s	1.33 s	10	10, 13	1.31 s	13	10, 11, 13
13	1.30 s	1.32 s	9	10, 12	1.26 s	10, 12	10, 12
14	0.80 d (6.8)	0.83 d (6.8)	1, 7	1, 7, 8	0.82 d (6.8)	7, 8	1, 7, 8
15	1.94 s	1.94 s	3, 5	3, 5	1.94 s	3, 5	3, 4, 5

[a] Recorded in $CDCl_3$ at 400 MHz. Values in ppm (δ). *J* (in Hz) in parentheses.

NMR ($CDCl_3$, 100 MHz): δ 17.0 (C-14), 23.8 (C-15), 28.0 (C-6), 29.9 (C-12), 29.9 (C-13), 36.9 (C-1), 37.1 (C-7), 39.8 (C-8), 70.8 (C-4), 70.9 (C-11), 73.5 (C-5), 125.7 (C-9), 131.8 (C-2), 132.7 (C-3), 139.7 (C-10); ESIMS *m/z* 277 $[M+Na]^+$; HRESIMS *m/z* 277.1782 $[M+Na]^+$ (calcd for $C_{15}H_{26}O_3Na$, 277.1780).

6α-Hydroxycurcumanolide A (**5**): colourless oil; $[\alpha]_D^{25}$ − 31.4 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 233 (4.01) nm; IR (neat) ν_{max} 3420 (OH), 1735 (C═O) cm^{-1}; ^{1}H NMR data, see Table 2; ^{13}C NMR ($CDCl_3$, 100 MHz): δ 17.2 (C-8), 19.9 (C-12), 21.5 (C-4), 23.8 (C-15), 24.3 (C-14), 24.5 (C-16), 30.9 (C-7), 49.1 (C-9), 79.5 (C-6), 97.1 (C-5), 112.7 (C-11), 120.9 (C-3), 143.9 (C-13), 149.2 (C-10), 169.9 (C-2); ESIMS *m/z* 273 $[M+Na]^+$; HRESIMS *m/z* 273.1466 $[M+Na]^+$ (calcd for $C_{15}H_{22}O_3Na$, 273.1467).

1,10-Dehydro-10-deoxy-9-oxozedoarondiol (**6**): colourless oil; UV (MeOH) λ_{max} (log ε) 206 (4.25), 228 (3.97), 268 (4.05) nm; IR (neat) ν_{max} 3395 (OH), 1682 (C═O), 1651 (C═O) cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.21 (3H, s, H-14), 1.80 (1H, m, H-3), 1.85 (3H, s, H-15), 1.91 (3H, d, *J* = 1.2 Hz, H-12), 1.95 (1H, m, H-3), 2.05 (3H, d, *J* = 1.2 Hz, H-13), 2.10 (1H, br dd, *J* = 14.8, 12.4 Hz, H-6), 2.87 (1H, dd, *J* = 14.8, 1.6 Hz, H-6), 2.45 (1H, m, H-2), 2.54 (1H, br d, *J* = 12.4 Hz, H-5), 2.65 (1H, m, H-2); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 13.6 (C-15), 21.6 (C-14), 22.6 (C-12), 23.2 (C-13), 28.0 (C-6), 30.2 (C-2), 38.1 (C-3), 55.9 (C-5), 80.3 (C-4), 129.7 (C-7), 129.9 (C-10), 147.0 (C-11), 164.5 (C-1), 197.5 (C-9), 199.0 (C-8); ESIMS *m/z* 271 $[M+Na]^+$; HRESIMS *m/z* 271.1311 $[M+Na]^+$ (calcd for $C_{15}H_{20}O_3Na$, 271.1310).

2.4. Biological assay

The effect of the isolated compounds on neutrophil pro-inflammatory response was evaluated by monitoring the inhibition of superoxide anion generation and the release of elastase in fMLP/CB-activated human neutrophils in a concentration-dependent manner.

2.4.1. Preparation of human neutrophils

Human neutrophils from venous blood of healthy, adult volunteers (20–28 years old) were isolated using a standard method of dextran sedimentation prior to centrifugation in a Ficoll Hypaque gradient and hypotonic lysis of erythrocytes (Boyum, 1968). Purified neutrophils containing >98% viable cells, as determined by the trypan blue exclusion method (Jauregui et al., 1981), were resuspended in a Ca^{2+}-free HBSS buffer at pH 7.4 and were maintained at 4 °C prior to use.

2.4.2. Measurement of superoxide anion generation

Measurement of superoxide anion generation was based on the SOD-inhibitable reduction of ferricytochrome *c* (Babior, Kipnes, & Curnutte, 1973; Hwang, Leu, Kao, Tang, & Chang, 2006). In brief, after supplementation with 0.5 mg/ml ferricytochrome *c* and 1 mM Ca^{2+}, neutrophils (6×10^5/ml) were equilibrated at 37 °C for 2 min and incubated with different concentrations of compounds or DMSO (as control) for 5 min. Cells were incubated with cytochalasin B (1 μg/ml) for 3 min prior to the activation with

Table 2
^{1}H NMR data of **5** and **13**.[a]

Position	13	5		
	δ_H *J* (Hz)	δ_H *J* (Hz)	NOE	HMBC
4	2.47 br s	2.48 br s	12, 15, 16	2, 6, 13
6	2.33 m			
7	1.18 m	1.34 m	8	5, 9
	1.92 m	2.08 m	8, 16	5, 9, 16
8	1.65 m	1.67 m	7, 9	5
	1.80 m	1.81 m	7, 12	5, 6
9	2.82 br dd (11.8, 8.8)	2.83 br dd (11.8, 8.8)	8, 11	6, 11, 12
11	4.76 br s	4.75 br s	9	9, 10, 12
	4.95 br s	4.95 br s	12	9, 12
12	1.73 br s	1.74 br s	4, 8, 11	9, 11
14	2.24 br s	2.24 br s	15	3, 15
15	1.84 br s	1.85 br s	4, 14	3, 13, 14
16	0.87 d (6.8)	1.29 s	4, 7	5, 6, 7

[a] Recorded in $CDCl_3$ at 400 MHz. Values in ppm (δ). *J* (in Hz) in parentheses.

100 nM formyl-L-methionyl-L-leucyl-L-phenylalanine for 10 min. Changes in absorbance with the reduction of ferricytochrome *c* at 550 nm were continuously monitored in a double-beam, six-cell positioner spectrophotometer with constant stirring (Hitachi U-3010, Tokyo, Japan). Calculations were based on differences in the reactions with and without SOD (100 U/ml) divided by the extinction coefficient for the reduction of ferricytochrome *c* (ε = 21.1/mM/10 mm).

2.4.3. Measurement of elastase release

Degranulation of azurophilic granules was determined by measuring elastase release as described previously (Hwang et al., 2006). Experiments were performed using MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide as the elastase substrate. Briefly, after supplementation with MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide (100 μM), neutrophils (6×10^5/ml) were equilibrated at 37 °C for 2 min and incubated with compounds for 5 min. Cells were stimulated with fMLP (100 nM)/CB (0.5 μg/ml), and changes in absorbance at 405 nm were monitored continuously in order to assay elastase release. The results were expressed as the percent of elastase release in the fMLP/CB-activated, drug-free control system.

2.4.4. Xanthine oxidase assay

The $O_2^{\cdot-}$-scavenging ability of compounds was determined using xanthine/xanthine oxidase in a cell-free system, based on a previously described method (Hwang et al., 2009). After 0.1 mM xanthine was added to the assay buffer [50 mM Tris (pH 7.4), 0.3 mM WST-1, and 0.02 U/ml xanthine oxidase] for 15 min at 30 °C, the absorbance associated with the $O_2^{\cdot-}$-induced WST-1 reduction was measured at 450 nm.

3. Results and discussion

3.1. General

Chromatographic purification of the CH_2Cl_2-soluble fraction of a MeOH extract of rhizome of *C. longa* on a silica gel column and preparative thin-layer chromatography (TLC) afforded six new (**1–6**) (Fig. 1) and nineteen known compounds (**7–25**).

3.2. Structure elucidation of the new sesquiterpenes

Curculonone A (**1**) was isolated as colourless oil. Its molecular formula, $C_{15}H_{24}O_2$, was determined on the basis of the positive HRESIMS at *m/z* 259.1676 [M+Na]$^+$ (calcd 259.1674) and this was supported by the ^{1}H, ^{13}C, and DEPT NMR data. The IR spectrum showed the presence of OH (3435 cm^{-1}) and carbonyl (1670 cm^{-1}) groups. Comparison of the ^{1}H NMR data of **1** with those of (6*R*)-[(1*R*)-1,5-dimethylhex-4-enyl]-3-methylcyclohex-2-en-1-one (**11**) (Hagiwara et al., 2002) suggested that their structures were closely related, except that (*E*)-3-hydroxy-3-methylbut-1-enyl group [δ 1.30 (6H, s, H-12 and H-13), δ 5.58 (1H, dt, *J* = 15.6, 6.4 Hz, H-9), and δ 5.64 (1H, d, *J* = 15.6 Hz, H-10)] at C-8 of **1** replaced a C-8 3-methylbut-2-enyl group of **11**. This was supported by both HMBC correlations between H-9 (δ 5.58) and C-7 (δ 33.2) and C-11 (δ 70.3), and NOESY correlations between H-10 (δ 5.64) and both H-8 (δ 2.00) and H-12 (δ 1.30). The NOESY cross-peaks between H-1 (δ 2.17) and both H-7 (δ 2.40) and H$_\alpha$-6 (δ 1.77) suggested that H-1 and H-7 are on the α-side, and the methyl group at C-7 is on the β-side of **1**. Compound **1** showed similar CD Cotton effects [393 nm ([θ] − 285), 365 nm ([θ] 0), 345 nm ([θ] + 397), 305 nm ([θ] 0), 264 nm ([θ] − 1280), 251 nm ([θ] − 3475), and 241 nm ([θ] − 5655)] compared with the analogous sesquiterpene, (−)-oxojuvabione (Numata, Kawai, & Takahashi, 1990). Thus, **1** possessed a 1*R*,7*S*-configuration. The structure of **1** was thus elucidated as (*R*)-6-[(*S*,*E*)-6-hydroxy-6-methylhept-4-en-2-yl]-3-methylcyclohex-2-enone, named curculonone A. This structure was supported by ^{1}H–^{1}H COSY and NOESY (Fig. 2) experiments, and ^{13}C NMR assignments were confirmed by DEPT, HSQC, and HMBC (Fig. 2) techniques.

Curculonone B (**2**) was isolated as optically active colourless oil ($[\alpha]_D^{25}$ + 58.7), and HRESIMS gave an [M+Na]$^+$ ion at *m/z* 259.1673 consistent with molecular formula $C_{15}H_{24}O_2$. The IR spectrum indicated that OH (3440 cm^{-1}) and carbonyl

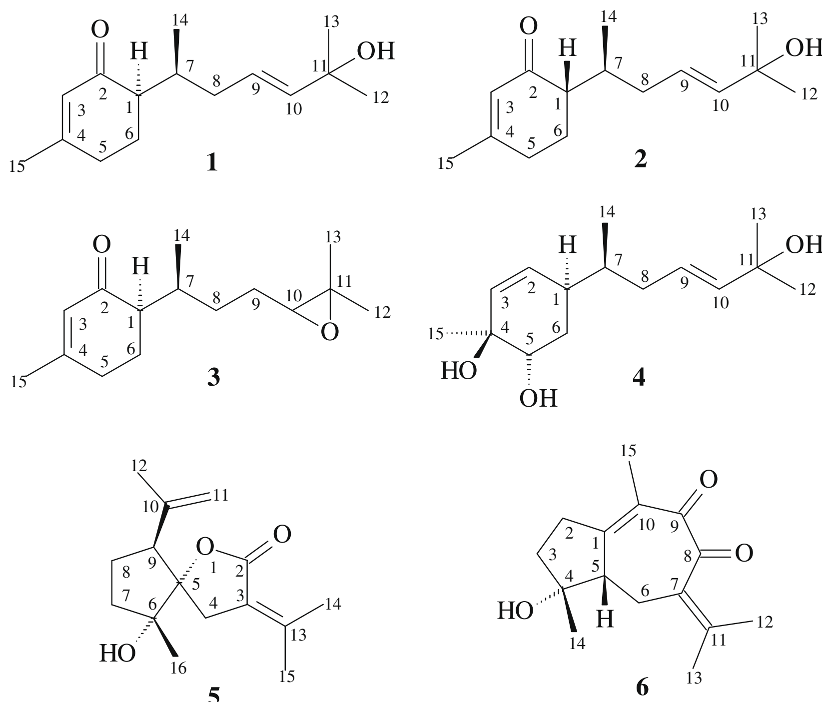

Fig. 1. The structures of new sesquiterpenes isolated from *C. longa*.

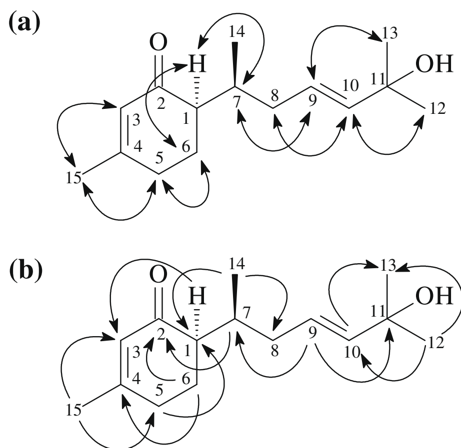

Fig. 2. NOESY (a) and HMBC (b) correlations of **1**.

(1670 cm^{-1}) groups were present. Comparison of the ^1H NMR data (Table 1) of **2** with those of **1** suggested that their structures were closely related, except that H_β-1 [δ 2.16 (1H, m)] of **2** replaced H_α-1 [δ 2.17 (1H, ddd, J = 12.0, 4.0, 4.0 Hz)] of **1**. This was supported by NOESY correlations between H_β-1 (δ 2.16) and H-14 (δ 0.83). Compound **2** showed a similar CD curve when compared to (+)-oxoepijuvabione (Numata et al., 1990) and the absolute configuration of **2** has to be 1*S*,7*S* (Numata et al., 1990). On the basis of the above data, the structure of **2** was elucidated as (*S*)-6-[(*S*,*E*)-6-hydroxy-6-methylhept-4-en-2-yl]-3-methylcyclohex-2-enone, named curculonone B, which was further confirmed by the ^1H–^1H COSY, NOESY (Table 1), DEPT, HSQC, and HMBC (Table 1) experiments.

Curculonone C (**3**) was isolated as colourless oil with molecular formula $C_{15}H_{24}O_2$ as determined by positive-ion HRESIMS, showing an $[M+Na]^+$ ion at *m/z* 259.1674 (calcd for $C_{15}H_{24}O_2Na$, 259.1674). The presence of a carbonyl group was revealed by a band at 1672 cm^{-1} in the IR spectrum, and was confirmed by the resonance at δ 201.0 in the ^{13}C NMR spectrum. The ^1H NMR data (Table 1) of **3** was similar to that of **2**, except that the (3,3-dimethyloxiran-2-yl)methyl group [δ 1.26 (3H, s, H-13), δ 1.31 (3H, s, H-12), δ 1.50 (2H, m, H-9), and δ 2.71 (1H, br t, J = 6.2 Hz, H-10)] at C-8 of **3** replaced the C-8 (*E*)-3-hydroxy-3-methylbut-1-enyl group of **1**. This was supported by HMBC correlations between H-10 (δ 2.71) and both C-8 (δ 32.8) and C-12 (δ 24.9) and NOESY correlations between H-9 (δ 1.50) and both H-8 (δ 1.38) and H-10 (δ 2.71). The NOESY cross-peaks between H-1 (δ 2.16) and both H-7 (δ 2.35) and H_α-6 (δ 1.80) suggested that H-1 and H-7 are on the α-side, and the methyl group at C-7 is on the β-side of **1**. Compound **3** showed similar CD Cotton effects to those of **1**, and the absolute configuration of **3** has to be 1*R*,7*S*. On the basis of the evidence above, the structure of **3** was elucidated as (*R*)-6-[(2*S*)-4-(3,3-dimethyloxiran-2-yl)butan-2-yl]-3-methylcyclohex-2-enone, named curculonone C. This was further confirmed by ^1H–^1H COSY and NOESY (Table 1) experiments. The assignment of ^{13}C NMR resonances was confirmed by DEPT, HSQC and HMBC (Table 1) techniques.

Curculonone D (**4**) was obtained as colourless oil. The molecular formula $C_{15}H_{26}O_3$ was deduced from a sodium adduct ion at *m/z* 277.1782 $[M+Na]^+$ (calcd 277.1780) in the HRESI mass spectrum. The presence of hydroxy group was revealed by a band at 3390 cm^{-1} in the IR spectrum. The ^1H NMR data of **4** was similar to that of bisacurone (**8**) (Ohshiro et al., 1990), except that (*E*)-3-hydroxy-3-methylbut-1-enyl group [δ 1.31 (6H, s, H-12 and H-13), δ 5.60 (1H, dt, J = 15.6, 6.4 Hz, H-9), and δ 5.65 (1H, d, J = 15.6 Hz, H-10)] at C-8 of **4** replaced the C-8 3-methylbut-2-enoyl group of bisacurone (**8**) (Ohshiro et al., 1990). This was supported by HMBC correlations between H-9 (δ 5.60) and C-7 (δ 37.1) and C-11 (δ 70.9) and NOESY correlations between H-10 (δ 5.65) and both H-8 (δ 2.01) and H-12 (δ 1.31). The CD spectrum of **4** showed Cotton effect at 320 nm ([θ] = +518) very similar to that of bisacurone (Ohshiro et al., 1990), indicating identical absolute configurations. Thus, the structure of **4** was elucidated as (1*S*,2*S*,5*R*)-5-[(*S*,*E*)-6-hydroxy-6-methylhept-4-en-2-yl]-2-methylcyclohex-3-ene-1,2-diol, named curculonone D, which was confirmed by ^1H–^1H COSY, NOESY (Fig. 3), DEPT, HSQC, and HMBC (Fig. 3) experiments.

6α-Hydroxycurcumanolide A (**5**) had the molecular formula $C_{15}H_{22}O_3$ as indicated by the sodiated HRESIMS ion peak at *m/z* = 273.1466 $[M+Na]^+$ (calcd for $C_{15}H_{22}O_3Na$, 273.1467). Hydroxy and carbonyl groups were revealed by IR bands at 3420 and 1735 cm^{-1}, respectively. The ^1H NMR spectrum of **5** was similar to that of curcumanolide A (**13**) (Shiobara et al., 1985) except that the 6α-hydroxy group of **5** replaced H_α-6 of curcumanolide A (**13**). This was supported by the HMBC correlations between H-16 (δ 1.29) and C-5 (δ 97.1), C-6 (δ 79.5), and C-7 (δ 30.9). The NOESY cross-peaks between H-4 and both H-12 and H-16 suggested that the 6-methyl group, the C-9 prop-1-en-2-yl group, and the bond between C-4 and C-5 are on the β-side, and the hydroxy group at C-6 is on the α-side of **5**. Assignments of the carbon resonances of **5** were confirmed by DEPT, HSQC, and HMBC (Table 2) techniques. Based on the above data, the structure of **5** was elucidated as 6α-hydroxycurcumanolide A, which was confirmed by the ^1H–^1H COSY, and NOESY (Table 2) experiments.

1,10-Dehydro-10-deoxy-9-oxozedoarondiol (**6**) was isolated as colourless oil. The ESIMS of **6** afforded an $[M+Na]^+$ ion at *m/z* 271, implying a molecular formula of $C_{15}H_{20}O_3$, which was confirmed by HRESIMS. The IR spectrum showed the presence of OH (3395 cm^{-1}) and carbonyl (1682 and 1651 cm^{-1}) groups. Comparison of the ^1H NMR data of **6** with those of zedoarondiol (**12**) (Kouno & Kawano, 1985) suggested that their structures were closely related, except that 1,10-dehydro-10-deoxy-9-oxo moiety of **6** replaced the 10-hydroxy group of zedoarondiol (**12**). This was supported by HMBC correlations between H-15 (δ 1.85) and C-1 (δ 164.5), C-9 (δ 197.5), and C-10 (δ 129.9). NOESY correlations (Fig. 3) of **6** were observed between H-14 (δ 1.21) and H-5 (δ 2.54) suggested that the 4-methyl group and H-5 are on the β-side, and the 4-hydroxy group is on the α-side of **6**. The structure of **6** was thus elucidated as 1,10-dehydro-10-deoxy-9-oxozedoarondiol. This structure was supported by ^1H–^1H COSY and NOESY

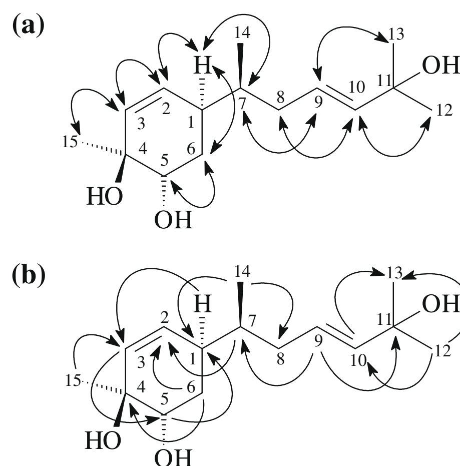

Fig. 3. NOESY (a) and HMBC (b) correlations of **4**.

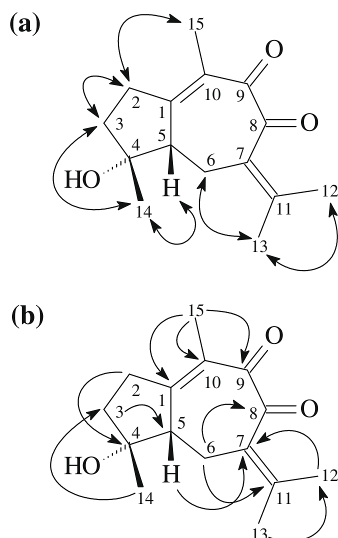

Fig. 4. NOESY (a) and HMBC (b) correlations of **6**.

(Fig. 4) experiments, and ^{13}C NMR assignments were confirmed by DEPT, HSQC, and HMBC (Fig. 4) techniques.

The known isolates were readily identified by a comparison of physical and spectroscopic data (UV, IR, ^{1}H NMR, $[\alpha]_D$, and MS) with corresponding authentic samples or literature values, and this included eight sesquiterpenes, (*S*)-(+)-*ar*-turmerone (**7**) (Mori, 2005), bisacurone (**8**) (Ohshiro et al., 1990), curlone (**9**) (Kiso et al., 1983), β-atlantone (**10**) (Itokawa et al., 1985), (6*R*)-[(1*R*)-1,5-dimethylhex-4-enyl]-3-methylcyclohex-2-en-1-one (**11**) (Hagiwara et al., 2002), zedoarondiol (**12**) (Kouno & Kawano, 1985), curcumanolide A (**13**) (Shiobara et al., 1985), and curcumanolide B (**14**) (Shiobara et al., 1985), three curcuminoids, curcumin (**15**) (Park et al., 2005), demethoxycurcumin (**16**) (Park et al., 2005), and bisdemethoxycurcumin (**17**) (Park et al., 2005), six benzenoids, vanillin (**18**) (Chen, Duh, & Chen, 2005), vanillic acid (**19**) (Chen, Chou, Peng, Chen, & Yang, 2007), (*E*)-ferulic acid (**20**) (Machida & Kikuchi, 1992), (*Z*)-ferulic acid (**21**) (Machida & Kikuchi, 1992), (*E*)-4-(4-hydroxy-3-methoxyphenyl)but-3-en-2-one (**22**) (Ayer & Singer, 1980), and 1,5-bis(4-hydroxy-3-methoxyphenyl)-(1*E*,4*E*)-1,4-pentadien-3-one (**23**) (Masuda et al., 1993), and a mixture of β-sitosterol (**24**) (Chen, Lin, Liao, & Shieh, 2007) and stigmasterol (**25**) (Chen et al., 2007).

Neutrophils accumulate at sites of inflammation and immunological reaction in response to locally existing chemotactic mediators. The bacterial *N*-formyl peptides, such as formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), are some of the first identified and most potent chemoattractants for neutrophils (Le et al., 2002). When fMLP was used as a stimulant, cytochalasin B (CB), a priming agent, was incubated for 3 min before activation by peptide (fMLP/CB). In this study, the effects on neutrophil pro-inflammatory responses of compounds isolated from the rhizome of *C. longa* were evaluated by suppressing fMLP/CB-induced superoxide radical anion ($O_2^{\cdot -}$) generation and elastase release by human neutrophils. The inhibitory activity data on neutrophil pro-inflammatory responses are shown in Table 3. Diphenyleneiodonium and phenylmethylsulfonyl fluoride were used as positive controls for $O_2^{\cdot -}$ generation and elastase release, respectively. From the results of our biological tests, the following conclusions can be drawn: (a) Curculonone A (**1**), curculonone B (**2**), 6α-hydroxycurcumanolide A (**5**), zedoarondiol (**12**), curcumin (**15**), demethoxycurcumin (**16**), and 1,5-bis(4-hydroxy-3-methoxyphenyl)-(1*E*,4*E*)-1,4-pentadien-3-one (**23**) exhibited inhibitory activities ($IC_{50} \leqslant 18.22$ μM) on human neutrophil $O_2^{\cdot -}$ generation. (b) 6α-Hydroxycurcumanolide A (**5**), zedoarondiol (**12**), curcumanolide A (**13**), curcumanolide B (**14**), curcumin (**15**), demethoxycurcumin (**16**), and 1,5-bis(4-hydroxy-3-methoxyphenyl)-(1*E*,4*E*)-1,4-pentadien-3-one (**23**) inhibited fMLP/CB-induced elastase release with IC_{50} values $\leqslant$ 14.28 μM. (c) Among the curcuminoid analogues (**15**–**17**), curcumin (**15**), with two feruloyl moieties, exhibited more effective inhibition than

Table 3
Inhibitory effects of **1**–**25** on superoxide radical anion generation and elastase release by human neutrophils in response to fMet-Leu-Phe/cytochalasin B.

Compound	IC_{50} (μM)[a]	
	Superoxide anion generation	Elastase release
Curculonone A (**1**)	16.06 ± 1.13	20.34 ± 4.66
Curculonone B (**2**)	18.22 ± 1.43	23.52 ± 3.66
Curculonone C (**3**)	24.61 ± 2.07	21.48 ± 0.89
Curculonone D (**4**)	>50	>50
6α-Hydroxycurcumanolide A (**5**)	17.50 ± 1.12	10.82 ± 1.64
1,10-Dehydro-10-deoxy-9-oxozedoarondiol (**6**)	>50	>50
(*S*)-(+)-*ar*-Turmerone (**7**)	>50	38.98 ± 18.47
Bisacurone (**8**)	>50	>50
Curlone (**9**)	>50	>50
β-Atlantone (**10**)	>50	>50
(6*R*)-[(1*R*)-1,5-dimethylhex-4-enyl]-3-methylcyclohex-2-en-1-one (**11**)	32.31 ± 3.52	36.20 ± 3.90
Zedoarondiol (**12**)	7.54 ± 0.95	12.66 ± 3.41
Curcumanolide A (**13**)	21.41 ± 1.49	11.70 ± 2.35
Curcumanolide B (**14**)	22.35 ± 1.65	12.55 ± 2.48
Curcumin (**15**)	7.71 ± 0.63	10.16 ± 3.72
Demethoxycurcumin (**16**)	15.02 ± 1.45	11.69 ± 0.38
Bisdemethoxycurcumin (**17**)	25.00 ± 3.02	17.62 ± 5.10
Vanillin (**18**)	>50	>50
Vanillic acid (**19**)	>50	>50
(*E*)-Ferulic acid (**20**)	23.45 ± 1.08	24.63 ± 6.03
(*Z*)-Ferulic acid (**21**)	25.64 ± 1.42	28.33 ± 5.82
(*E*)-4-(4-Hydroxy-3-methoxyphenyl)but-3-en-2-one (**22**)	>50	>50
1,5-bis(4-Hydroxy-3-methoxyphenyl)-(1*E*,4*E*)-1,4-pentadien-3-one (**23**)	16.30 ± 1.62	14.28 ± 0.52
Mixture of β-sitosterol (**24**) and stigmasterol (**25**)	>50	>50
Diphenyleneiodonium	1.72 ± 0.75	
Phenylmethylsulfonyl fluoride		200.5 ± 31.7

Diphenyleneiodonium and phenylmethylsulfonyl fluoride were used as positive control. Results are presented as average ± SEM (*n* = 4).
[a] Concentration necessary for 50% inhibition (IC_{50}).

Table 4
Antioxidant effects of compounds in a cell-free xanthine/xanthine oxidase system.

Compound	Conc.	WST-1 reduction (% of control)
Curculonone A (**1**)	50 μM	93.38 ± 2.88
Curculonone B (**2**)	50 μM	95.05 ± 2.13
Curculonone C (**3**)	50 μM	95.05 ± 1.03**
6α-Hydroxycurcumanolide A (**5**)	50 μM	98.74 ± 3.15
(*S*)-(+)-*ar*-turmerone (**7**)	50 μM	96.01 ± 1.01*
(6*R*)-[(1*R*)-1,5-dimethylhex-4-enyl]-3-methylcyclohex-2-en-1-one (**11**)	50 μM	94.78 ± 1.24*
Zedoarondiol (**12**)	50 μM	94.77 ± 2.43
Curcumanolide A (**13**)	50 μM	94.12 ± 2.45
Curcumanolide B (**14**)	50 μM	96.42 ± 1.97
Curcumin (**15**)	50 μM	84.06 ± 2.91**
Demethoxycurcumin (**16**)	50 μM	77.74 ± 2.99**
Bisdemethoxycurcumin (**17**)	50 μM	82.13 ± 0.59***
(*E*)-ferulic acid (**20**)	50 μM	92.26 ± 4.96
(*Z*)-ferulic acid (**21**)	50 μM	95.98 ± 3.13
1,5-bis(4-Hydroxy-3-methoxyphenyl)-(1*E*,4*E*)-1,4-pentadien-3-one (**23**)	50 μM	90.13 ± 2.19*
SOD	50 U/ml	8.17 ± 0.18***

Reduction of WST-1 was measured spectrophotometrically at 450 nm. All data are expressed as average ± SEM ($n = 3$).
* $p < 0.05$ compared with the control.
** $p < 0.01$ compared with the control.
*** $p < 0.001$ compared with the control.

analogues **16** and **17** against fMLP-induced $O_2^{\cdot -}$ generation and elastase release. (d) Zedoarondiol (**12**), with a 10-hydroxy group, showed strong inhibition against fMLP-induced $O_2^{\cdot -}$ generation and elastase release, but its analogue, 1,10-dehydro-10-deoxy-9-oxozedoarondiol (**6**), with the 1,10-dehydro-10-deoxy-9-oxo moiety, was inactive. (e) Zedoarondiol (**12**) was the most effective among these compounds, with an IC_{50} value of 7.54 ± 0.95 μM against fMLP-induced superoxide anion generation. (f) Curcumin (**15**) exhibited the most effective inhibition among the isolates, with IC_{50} value of 10.16 ± 3.72 μM against fMLP-induced elastase release. (g) None of these compounds significantly scavenged $O_2^{\cdot -}$ formation (IC_{50} > 50 μM) in a cell-free system (Table 4). Superoxide dismutase (SOD) was used as positive control. These data rule out the possibility that the inhibitory effects of these compounds on $O_2^{\cdot -}$ release occur through directly scavenging of $O_2^{\cdot -}$.

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