

Article

New Coumarin Derivatives and Other Constituents from the Stem Bark of *Zanthoxylum avicennae*: Effects on Neutrophil Pro-Inflammatory Responses

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Abstract: Three new coumarin derivatives, 8-formylalloxanthoxyletin (**1**), avicennone (**2**), and (*Z*)-avicennone (**3**), have been isolated from the stem bark of *Zanthoxylum avicennae* (*Z. avicennae*), together with 15 known compounds (**4–18**). The structures of these new compounds were determined through spectroscopic and MS analyses. Compounds **1**, **4**, **9**, **12**, and **15** exhibited inhibition (half maximal inhibitory concentration (IC_{50}) values $\leq 7.65 \mu\text{g/mL}$) of superoxide anion generation by human neutrophils in response to formyl-L-methionyl-L-leucyl-L-phenylalanine/cytochalasin B (fMLP/CB). Compounds **1**, **2**, **4**, **8** and **9** inhibited fMLP/CB-induced elastase release with IC_{50} values $\leq 8.17 \mu\text{g/mL}$. This investigation reveals bioactive isolates (especially **1**, **2**, **4**, **8**, **9**, **12** and **15**) could be further developed as potential candidates for the treatment or prevention of various inflammatory diseases.

Keywords: *Zanthoxylum avicennae*; Rutaceae; coumarin; structure elucidation; anti-inflammatory activity

1. Introduction

Zanthoxylum avicennae (Lam.) DC. (Rutaceae) is an evergreen shrub distributed in Vietnam, Philippines, southern China, and Taiwan [1]. Neolignans [2], alkaloids [2–4], coumarins [3,4], flavonoids [3], benzenoids [3,4], and their derivatives were isolated from this plant in previous studies. Many of these compounds were found to exhibit anti-inflammatory [2,3], anti-platelet aggregation [5], and anti-hepatitis B virus (HBV) [6] activities.

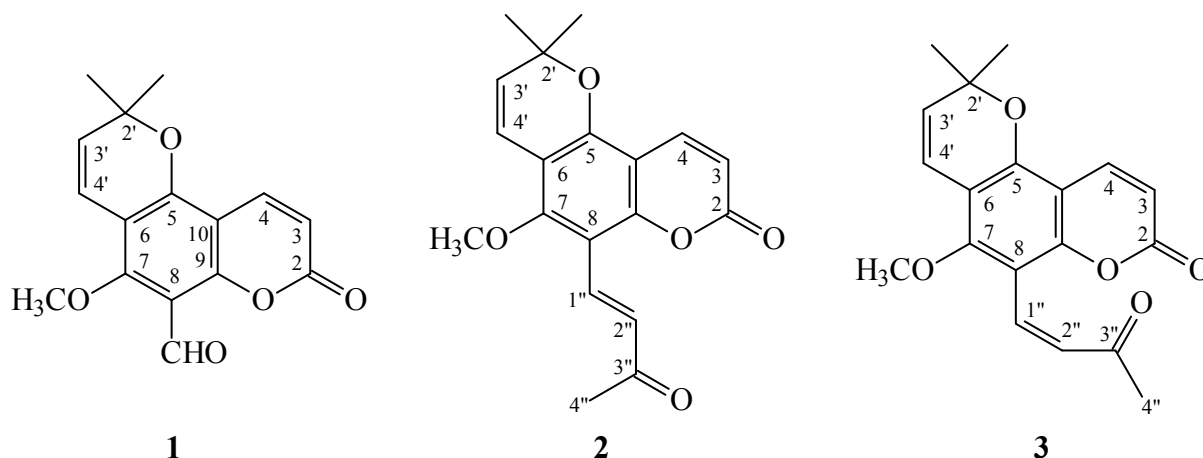


Figure 1. The chemical structures of new compounds **1–3** isolated from *Z. avicennae*.

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 *Z. avicennae* has been found to be an active species. The MeOH extract of stem bark of *Z. avicennae* showed potent inhibitory effects on superoxide anion generation and elastase release by human neutrophils in response

to formyl-L-methionyl-L-leucyl-L-phenylalanine/cytochalasin B (fMLP/CB). Figure 1 illustrates the structures of three new coumarins, 8-formylalloxanthoxyletin (**1**), avicennone (**2**), and (*Z*)-avicennone (**3**). Fifteen known compounds (**4–18**), have been isolated and identified from the stem bark of *Z. avicennae* and their structures are depicted in Figure 2.

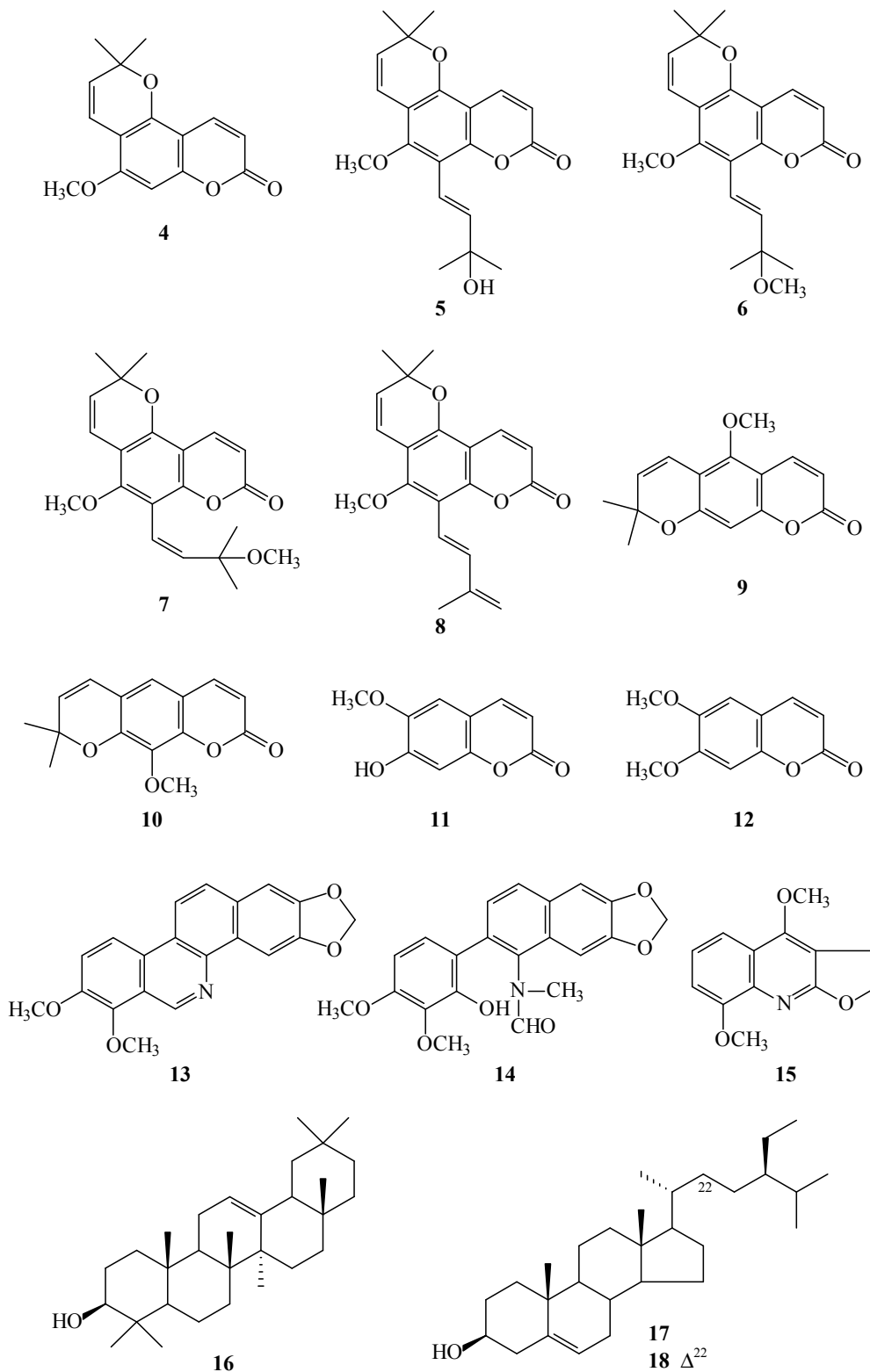


Figure 2. The chemical structures of known compounds **4–18** isolated from *Z. avicennae*.

This paper describes the structural elucidation of the compounds numbered **1** through **3**, and the inhibitory activities of all isolates on superoxide generation and elastase release by neutrophils.

2. Results

Chromatographic purification of the EtOAc-soluble fraction of a MeOH extract of stem bark of *Z. avicennae* on a silica gel column and preparative thin-layer chromatography (TLC) afforded three new (**1–3**) and fifteen known compounds (**4–18**).

8-Formylalloxanthoxyletin (**1**) was isolated as amorphous powder with molecular formula $C_{16}H_{14}O_5$ as determined by positive-ion high-resolution electrospray ionization (HR-ESI-MS), showing an $[M + Na]^+$ ion at m/z 309.0739 (calculated for $C_{16}H_{14}O_5Na$, 309.0739). The presence of carbonyl groups was revealed by the bands at 1693, 1729 cm^{-1} in the Infrared (IR) spectrum, and was confirmed by the resonances at δ 159.4, 185.7 in the ^{13}C NMR spectrum. The 1H NMR spectrum of **1** showed the presence of a 2,2-dimethyl-2*H*-pyran moiety [δ 1.53 (6H, s, Me-2' \times 2), 5.70, 6.64 (each 1H, each d, $J = 10.5$ Hz, H-3' and H-4')], a methoxy group [δ 3.92 (3H, s, OMe-7)], a formyl group [δ 10.53 (1H, s, CHO-8)], and the typical H-3 and H-4 protons of the coumarin nucleus [δ 6.32, 8.00 (each 1H, each d, $J = 10.0$ Hz, H-3 and H-4)]. The 1H NMR spectrum of **1** was similar to those of alloxanthoxyletin (**4**) [7], except that the 8-formyl group [δ 10.53 (1H, s)] of **1** replaced H-8 of alloxanthoxyletin (**4**). This was supported by HMBC correlation observed between CHO-8 (δ 10.53) and C-7 (δ 160.2), C-8 (δ 110.7), and C-9 (δ 157.5). The full assignment of 1H and ^{13}C NMR resonances was confirmed by 1H – 1H correlation spectroscopy (COSY), nuclear Overhauser effect spectrometry (NOESY) (Figure 3), Distortionless Enhancement by Polarization Transfer (DEPT), heteronuclear single-quantum coherence (HSQC), and heteronuclear multiple-bond correlation (HMBC) (Figure 3) techniques. According to the evidence above, the structure of **1** was elucidated as 5-methoxy-2,2-dimethyl-8-oxo-2,8-dihydropyrano [2,3-*f*]chromene-6-carbaldehyde, named 8-formylalloxanthoxyletin.

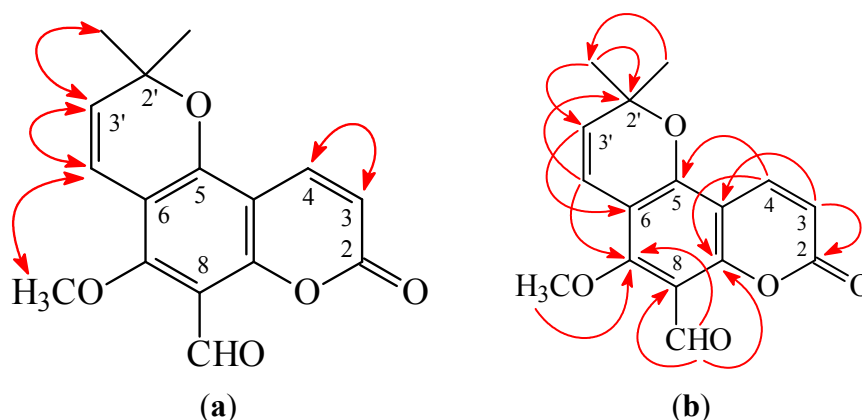


Figure 3. Key nuclear Overhauser effect spectrometry (NOESY) (a) and heteronuclear multiple-bond correlation (HMBC) (b) correlations of **1**.

(*E*)-Avicennone (**2**) was obtained as amorphous powder. Its molecular formula, $C_{19}H_{18}O_5$, was determined on the basis of the positive HRESIMS at m/z 349.1056 $[M + Na]^+$ (calcd 349.1052) and supported by the 1H , ^{13}C , and DEPT NMR data. The presence of carbonyl groups was revealed by the bands at 1695 and 1734 cm^{-1} in the IR spectrum, and was confirmed by the resonances at δ 160.0

and 199.8 in the ^{13}C NMR spectrum. The ^1H NMR spectrum of **2** displayed the presence of a 2,2-dimethyl-2*H*-pyran moiety [δ 1.51 (6H, s, Me-2' \times 2), 5.70, 6.59 (each 1H, each d, $J = 10.2$ Hz, H-3' and H-4')], a methoxy group [δ 3.81 (3H, s, OMe-7)], an (*E*)-3-oxobut-1-enyl group [δ 2.42 (3H, s, Me-3''), 7.32, 7.83 (each 1H, each d, $J = 16.5$ Hz, H-2'' and H-1'')], and the typical H-3 and H-4 protons of the coumarin nucleus [δ 6.30, 8.02 (each 1H, each d, $J = 9.6$ Hz, H-3 and H-4)]. The ^1H NMR data of **2** were similar to 8-formylalloxanthoxyletin (**1**), except that the (*E*)-3-oxobut-1-enyl group at C-8 of **2** replaced 8-formyl group of 8-formylalloxanthoxyletin (**1**). This was supported by the NOESY correlations between H-1'' (δ 7.83) and H-4'' (δ 2.42) and OMe-7 (δ 3.81) and by the HMBC correlation between H-1'' (δ 7.83) and C-7 (δ 159.2), C-8 (δ 107.5), C-9 (δ 154.1), and C-3'' (δ 199.8) of **2**. In addition, the (*E*)-configuration of **2** was established by the following evidences: (a) The larger coupling constant ($J = 16.5$ Hz) between H-1'' and H-2'' of **2**; (b) The NOESY correlations were observed between H-1'' (δ 7.83) and H-4'' (δ 2.42) (Figure 3). Thus, (*E*)-avicennone was elucidated as structure **2**. This structure was confirmed by the ^1H - ^1H COSY, NOESY (Figure 4), DEPT, HSQC, and HMBC techniques (Figure 4).

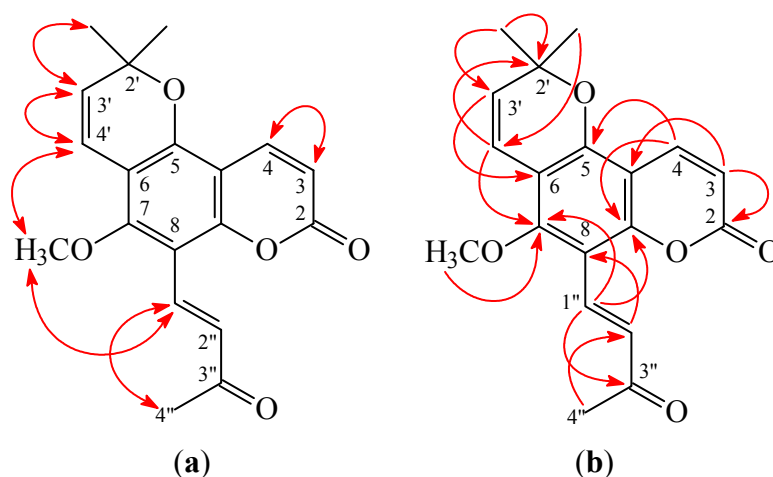


Figure 4. Key NOESY (a) and HMBC (b) correlations of **2**.

(*Z*)-Avicennone (**3**) was isolated as amorphous powder. The molecular formula $\text{C}_{19}\text{H}_{18}\text{O}_5$ was deduced from a sodium adduct ion at m/z 349.1054 [$\text{M} + \text{Na}]^+$ (calcd 349.1052) in the HR-ESI mass spectrum. The presence of carbonyl groups was revealed by the bands at 1694, 1734 cm^{-1} in the IR spectrum. The ^1H NMR spectrum of **3** illustrated the presence of a 2,2-dimethyl-2*H*-pyran moiety [δ 1.49 (6H, s, Me-2' \times 2), 5.64, 6.56 (each 1H, each d, $J = 10.2$ Hz, H-3' and H-4')], a methoxy group [δ 3.72 (3H, s, OMe-7)], an (*Z*)-3-oxobut-1-enyl group [δ 2.23 (3H, s, Me-3''), 6.40, 6.79 (each 1H, each d, $J = 12.6$ Hz, H-2'' and H-1'')], and the typical H-3 and H-4 protons of the coumarin nucleus [δ 6.23, 7.99 (each 1H, each d, $J = 9.6$ Hz, H-3 and H-4)]. The ^1H and ^{13}C NMR data of **3** were similar to those of (*E*)-avicennone (**2**), except that the (*Z*)-3-oxobut-1-enyl group [δ_{H} 2.23 (3H, s, Me-3''), 6.40, 6.79 (each 1H, each d, $J = 12.6$ Hz, H-2'' and H-1''); δ_{C} 29.7 (C-4''), 128.6 (C-1''), 132.3 (C-2''), 200.6 (C-3'')] at C-8 of **3** replaced the (*E*)-3-oxobut-1-enyl group at C-8 of (*E*)-avicennone (**2**). This was supported by the coupling constant ($J = 12.6$ Hz) between H-1'' and H-2'' of **3** and by the NOESY correlations between H-1'' (δ 6.79) and H-2'' (δ 6.40). According to the above data, the structure of **3** was

elucidated as (*Z*)-avicennone. This was supported by ^1H - ^1H COSY and NOESY (Figure 5) experiments, and ^{13}C NMR assignments were confirmed by DEPT, HSQC, and HMBC (Figure 5) techniques.

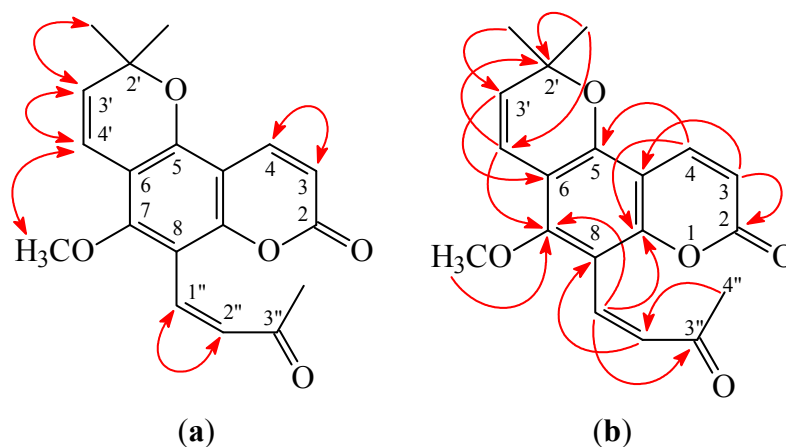


Figure 5. Key NOESY (a) and HMBC (b) correlations of **3**.

The known isolates were readily identified by a comparison of physical and spectroscopic data (UV, IR, ^1H NMR, $[\alpha]_D$, and MS) with corresponding authentic samples or literature values, and this included nine coumarins, alloxanthoxyletin (**4**) [7], avicennol (**5**) [8], avicennol methyl ether (**6**) [9], *cis*-avicennol methyl ether (**7**) [9], avicennin (**8**) [8] [9], xanthoxyletin (**9**) [7], luvangetin (**10**) [10], scopoletin (**11**) [11], and aesculetin dimethyl ether (**12**) [12]; two benzo[*c*]phenanthridine derivatives, norchelerythrine (**13**) [13] and arnottianamide (**14**) [14]; a furoquinoline, γ -fagarine (**15**) [14]; a triterpene, β -amyrin (**16**) [15]; and two steroids, β -sitosterol (**17**) [16] and stigmasterol (**18**) [16].

Granule proteases (e.g., elastase, cathepsin G, and proteinase-3) and reactive oxygen species (ROS) (e.g., superoxide anion ($\text{O}_2^{\cdot-}$) and hydrogen peroxide) produced by human neutrophils are involved in the pathogenesis of a variety of inflammatory diseases. The effects on neutrophil pro-inflammatory responses of compounds isolated from the stem bark of *Z. avicennae* were evaluated by suppressing fMet-Leu-Phe/cytochalasin B (fMLP/CB)-induced superoxide anion ($\text{O}_2^{\cdot-}$) generation and elastase release by human neutrophils. The inhibitory activity data on neutrophil pro-inflammatory responses are summarized in Table 1. Diphenylethylidene and phenylmethylsulfonyl fluoride were used as positive controls for $\text{O}_2^{\cdot-}$ generation and elastase release, respectively. From the results of our biological tests, the following conclusions can be drawn: (a) 8-Formylalloxanthoxyletin (**1**), alloxanthoxyletin (**4**), xanthoxyletin (**9**), aesculetin dimethyl ether (**12**), and γ -fagarine (**15**) exhibited potent inhibition ($\text{IC}_{50} \leq 7.65 \mu\text{g/mL}$) of superoxide anion ($\text{O}_2^{\cdot-}$) generation by human neutrophils in response to fMLP/CB; (b) 8-Formylalloxanthoxyletin (**1**), (*E*)-avicennone (**2**), alloxanthoxyletin (**4**), avicennin (**8**), and xanthoxyletin (**9**) exhibited potent inhibition ($\text{IC}_{50} \leq 8.17 \mu\text{g/mL}$) against fMLP-induced elastase release; (c) Among the coumarins with a 2,2-dimethyl-2*H*-pyran moiety at C-6,7, xanthoxyletin (**9**) (with a 5-methoxy group) exhibited more effective inhibition than its analogue, luvangetin (**10**) (with a 8-methoxy group) against fMLP-induced $\text{O}_2^{\cdot-}$ generation and elastase release; (d) Among the coumarins (**1–8**) with a 7-methoxy group and a 2,2-dimethyl-2*H*-pyran moiety at C-5,6, 8-formylalloxanthoxyletin (**1**) (with a 8-formyl group) and alloxanthoxyletin (**4**) (without any substituted group at C-8) exhibited more effective inhibition than its analogues (**2**, **3**, and **5–8**) against fMLP-induced $\text{O}_2^{\cdot-}$ generation and elastase release; (e) Alloxanthoxyletin (**4**) and xanthoxyletin (**9**) were the most effective among these

compounds, with IC₅₀ values of 1.47 ± 0.53 and 1.47 ± 0.41 µg/mL, respectively, against fMLP-induced superoxide anion generation; and (f) 8-Formylalloxanthoxyletin (**1**) exhibited the most effective among the isolates, with IC₅₀ value of 2.59 ± 0.52 µg/mL, against fMLP-induced elastase release.

Table 1. Inhibitory effects of compounds **1–18** from the stem bark of *Z. avicennae* on superoxide radical anion generation and elastase release by human neutrophils in response to fMet-Leu-Phe/cytochalasin B ^a.

Compounds	Superoxide Anion	Elastase
	IC ₅₀ [µg/mL] ^b or (Inh %) ^c	
8-Formylalloxanthoxyletin (1)	4.60 ± 0.83 ^d	2.59 ± 0.52 ^d
(<i>E</i>)-Avicennone (2)	(24.4 ± 5.1) ^e	8.17 ± 1.61 ^d
(<i>Z</i>)-Avicennone (3)	(18.0 ± 3.1)	(40.2 ± 4.5) ^f
Alloxanthoxyletin (4)	1.47 ± 0.53 ^d	3.43 ± 0.63 ^d
Avicennol (5)	(27.6 ± 4.8) ^e	10.9 ± 1.4 ^d
Avicennol methyl ether (6)	(28.6 ± 1.8) ^d	(6.31 ± 3.50)
<i>cis</i> -Avicennol methyl ether (7)	(23.2 ± 2.5) ^e	(6.51 ± 1.22) ^f
Avicennin (8)	(38.5 ± 4.2) ^d	6.21 ± 1.60 ^d
Xanthoxyletin (9)	1.47 ± 0.41 ^d	4.18 ± 0.73 ^d
Luvangetin (10)	(29.0 ± 3.7) ^e	(5.28 ± 1.30) ^f
Aesculetin dimethyl ether (11)	7.65 ± 1.62	(45.8 ± 5.1)
Scopoletin (12)	(25.6 ± 4.8)	(43.7 ± 3.2)
Norchelerythrine (13)	(12.1 ± 2.2) ^e	(23.8 ± 4.5) ^e
Arnottianamide (14)	(22.8 ± 4.2)	(16.5 ± 5.1)
γ-Fagarine (15)	6.85 ± 0.46 ^f	(21.3 ± 3.5)
β-Amyrin (16)	(8.75 ± 4.14)	(2.62 ± 1.61)
Mixture of β-Sitosterol (17) and Stigmasterol (18)	(1.25 ± 1.03)	(2.24 ± 1.14)
Diphenyleneiodonium ^g	0.53 ± 0.22 ^d	–
Phenylmethylsulfonyl fluoride ^g	–	34.2 ± 5.6 ^d

^a Results are presented as averages ± SEM (*n* = 4); ^b Concentration necessary for 50% inhibition (IC₅₀). If IC₅₀ value of compound was <10 µg/mL, it was displayed as IC₅₀ [µg/mL]; ^c Percentage of inhibition (Inh %) at 10 µg/mL. If IC₅₀ value of compound was ≥10 µg/mL, it was shown as (Inh %) at 10 µg/mL; ^d *p* < 0.001 compared with the control; ^e *p* < 0.01 compared with the control; ^f *p* < 0.05 compared with the control; ^g Diphenyleneiodonium and phenylmethylsulfonyl were used as positive controls for superoxide anion generation and elastase release, respectively.

3. Discussion

Eighteen compounds, including three new coumarins **1–3**, were isolated from the stem bark of *Z. avicennae*. Known compounds **4**, **7**, **9**, **14**, **16**, and **18** were obtained from this plant for the first time. The structures of these compounds were established on the basis of spectroscopic data. Discovery of more new coumarins from the genus *Zanthoxylum* may not only provide more structure-activity data of coumarins, but may also contribute to enhancing our understanding of the taxonomy and evolution of the genus *Zanthoxylum*.

Granule proteases (e.g., elastase, cathepsin G) and reactive oxygen species (ROS) (e.g., superoxide anion (O₂^{•-}), hydrogen peroxide) and produced by human neutrophils contribute to the pathogenesis of

inflammatory diseases. Inhibition of the inappropriate activation of neutrophils by drugs has been proposed as a way to ameliorate inflammatory diseases. Based on the results of our biological tests (Table 1), xanthoxyletin (**9**), alloxanthoxyletin (**4**), and 8-formylalloxanthoxyletin (**1**) were the most effective among these compounds, with IC_{50} values of 1.47 ± 0.41 , 1.47 ± 0.53 , and 4.60 ± 0.83 $\mu\text{g/mL}$, respectively, against fMLP-induced superoxide anion generation. 8-Formylalloxanthoxyletin (**1**), alloxanthoxyletin (**4**), and xanthoxyletin (**9**) exhibited the most effective among the isolates, with IC_{50} values of 2.59 ± 0.52 , 3.43 ± 0.63 , and 4.18 ± 0.73 $\mu\text{g/mL}$, respectively, against fMLP-induced elastase release. Our study suggests *Z. avicennae* and its isolates (especially **1**, **4**, and **9**) could be further developed as potential candidates for the treatment or prevention of various inflammatory diseases. More experiments should be performed to deduce the action modes of these compounds.

4. Experimental Section

4.1. Ethics Statement

Blood was taken from healthy human donors (20–30 years old) by venipuncture, using a protocol (No. 102-1595A3) approved by the Institutional Review Board at Chang Gung Memorial Hospital. All donors gave written consent. The Medical Ethics Committee of Chang Gung Memorial Hospital approved this consent procedure.

4.2. General Experimental Procedures

Melting points were determined on a Yanaco micro-melting point apparatus and were uncorrected. Optical rotations were measured using a Jasco DIP-370 polarimeter (Japan Spectroscopic Corporation, Tokyo, Japan) in CHCl_3 . Ultraviolet (UV) spectra were obtained on a Jasco UV-240 spectrophotometer (Japan Spectroscopic Corporation). Infrared (IR) spectra (neat or KBr) were recorded on a Perkin Elmer 2000 FT-IR spectrometer (Perkin Elmer Corporation, Norwalk, CT, USA). Nuclear magnetic resonance (NMR) spectra, including correlation spectroscopy (COSY), nuclear Overhauser effect spectrometry (NOESY), heteronuclear multiple-bond correlation (HMBC), and heteronuclear single-quantum coherence (HSQC) experiments, were acquired using Varian INOVA-500 or Varian VNMRS-600 spectrometer (Varian Inc., Palo Alto, CA, USA) operating at 500 or 600 MHz (^1H) and 125 or 150 MHz (^{13}C), respectively, with chemical shifts given in ppm (δ) using tetramethylsilane (TMS) as an internal standard. Electrospray ionisation (ESI) and high-resolution electrospray ionization (HRESI)-mass spectra were recorded on a Bruker APEX II (Bruker, Bremen, Germany) or a VG Platform Electrospray ESI/MS mass spectrometer (Fison, Villeurbanne, France). Silica gel (70–230, 230–400 mesh, Merck, Darmstadt, Germany) was used for column chromatography (CC). Silica gel 60 F-254 (Merck, Darmstadt, Germany) was used for thin-layer chromatography (TLC) and preparative thin-layer chromatography (PTLC).

4.3. Plant Material

The stem bark of *Z. avicennae* was collected from Yanpu, Pingtung County, Taiwan, in June 2006 and identified by Jih-Jung Chen. A voucher specimen (ZA-200606) was deposited in the Department of Pharmacy, Tajen University, Pingtung, Taiwan.

4.4. Extraction and Isolation

The dried stem bark (8.75 kg) of *Z. avicennae* was pulverized and extracted three times with MeOH (40 L each) for 3 days. The MeOH extracts were concentrated under reduced pressure at 35 °C, and the residue (550 g) was partitioned between EtOAc and H₂O (1:1). The EtOAc layer was concentrated to give a residue (fraction A, 302.8 g). The water layer was further extracted with *n*-BuOH, and the *n*-BuOH-soluble part (fraction B, 91.1 g) and the water-solubles (fraction C, 127.3 g) were separated. Fraction A (115 g) was chromatographed on silica gel (70–230 mesh, 5.2 kg), eluting with CH₂Cl₂, gradually increasing the polarity with MeOH to give 14 fractions: A1 (10 L, CH₂Cl₂), A2 (4 L, CH₂Cl₂/MeOH, 99:1), A3 (5 L, CH₂Cl₂/MeOH, 95:1), A4 (5 L, CH₂Cl₂/MeOH, 90:1), A5 (2 L, CH₂Cl₂/MeOH, 80:1), A6 (3 L, CH₂Cl₂/MeOH, 70:1), A7 (4 L, CH₂Cl₂/MeOH, 50:1), A8 (7.5 L, CH₂Cl₂/MeOH, 30:1), A9 (2 L, CH₂Cl₂/MeOH, 20:1), A10 (4 L, CH₂Cl₂/MeOH, 10:1), A11 (4 L, CH₂Cl₂/MeOH, 5:1), A12 (3 L, CH₂Cl₂/MeOH, 3:1), A13 (2 L, CH₂Cl₂/MeOH, 1:1), A14 (2 L, MeOH). Fraction A2 (11.8 g) was chromatographed further on silica gel (230–400 mesh, 535 g) eluting with *n*-hexane/EtOAc (3:1–0:1) to give 14 fractions (each 500 mL, A2-1–A2-14). Part (112 mg) of fraction A2-13 was purified by preparative TLC (silica gel, CH₂Cl₂/acetone, 30:1) to afford **12** (3.8 mg) ($R_f = 0.70$). Part (96 mg) of fraction A2-14 was purified by preparative TLC (silica gel, CH₂Cl₂/acetone, 25:1) to obtain **15** (3.3 mg) ($R_f = 0.60$). Fraction A4 (9.1 g) was chromatographed further on silica gel (230–400 mesh, 425 g) eluting with *n*-hexane/EtOAc (3:1–0:1) to give 13 fractions (each 1 L, A4-1–A4-13). Fraction A4-1 (273 mg) was purified by CC (silica gel, *n*-hexane/acetone, 2:1–0:1) to afford 11 subfractions (each 200 mL, A4-1-1–A4-1-11). Fraction A4-1-9 (33 mg) was purified by preparative TLC (silica gel, CHCl₃/EtOAc, 5:1) to give **11** (3.8 mg) ($R_f = 0.50$). Part (140 mg) of fraction A4-7 was purified by preparative TLC (silica gel, CHCl₃/acetone, 40:1) to obtain **7** (3.7 mg) ($R_f = 0.47$). Part (148 mg) of fraction A4-9 was purified by preparative TLC (silica gel, *n*-hexane/EtOAc, 5:1) to yield **1** (3.5 mg) ($R_f = 0.24$). Fraction A5 (8.8 g) was chromatographed further on silica gel (230–400 mesh, 405 g) eluting with *n*-hexane/EtOAc (10:1–0:1) to give 21 fractions (each 1 L, A5-1–A5-21). Part (81 mg) of fraction A5-8 was purified by preparative TLC (silica gel, *n*-hexane/EtOAc, 3:1) to afford **16** (6.0 mg) ($R_f = 0.71$). Part (72 mg) of fraction A5-11 was purified by preparative TLC (silica gel, *n*-hexane/CHCl₃, 1:3) to yield **9** (20.1 mg) ($R_f = 0.33$). Fraction A5-14 (225 mg) was purified by CC (silica gel, CH₂Cl₂/acetone, 45:1) to afford 5 subfractions (each 200 mL, A5-14-1–A5-14-5). Fraction A5-14-2 (28 mg) was purified by preparative TLC (silica gel, *n*-hexane/EtOAc, 5:1) to give **4** (10.2 mg) ($R_f = 0.35$). Fraction A5-14-3 (67 mg) was purified by preparative TLC (silica gel, *n*-hexane/EtOAc, 5:1) to obtain **6** (40.1 mg) ($R_f = 0.30$). Part (118 mg) of fraction A5-17 was purified by preparative TLC (silica gel, *n*-hexane/EtOAc, 2:1) to afford **10** (77 mg) ($R_f = 0.40$). Fraction A6 (10.5 g) was chromatographed further on silica gel (230–400 mesh, 478 g) eluting with *n*-hexane/acetone (2:1–0:1) to give 11 fractions (each 500 mL, A6-1–A6-11). Part (125 mg) of fraction A6-1 was purified by preparative TLC (silica gel, CHCl₃/acetone, 80:1) to obtain **2** (3.9 mg) ($R_f = 0.87$) and **3** (2.8 mg) ($R_f = 0.88$). Fraction A7 (9.2 g) was chromatographed further on silica gel (230–400 mesh, 420 g) eluting with *n*-hexane/EtOAc (6:1–0:1) to give 17 fractions (each 500 mL, A7-1–A7-17). Part (136 mg) of fraction A7-7 was purified by preparative TLC (silica gel, *n*-hexane/EtOAc, 4:1) to obtain mixture of **17** and **18** (14.1 mg) ($R_f = 0.50$). Part (135 mg) of fraction A7-8 was purified by preparative TLC (silica gel, *n*-hexane/EtOAc, 3:1) to yield **8** (10.2 mg) ($R_f = 0.46$). Part (125 mg) of fraction A7-9 was purified by preparative TLC (silica gel, CHCl₃/EtOAc, 15:1) to give

5 (3.5 mg) ($R_f = 0.79$). Part (95 mg) of fraction A7-13 was purified by preparative TLC (silica gel, *n*-hexane/EtOAc, 2:1) to yield **13** (4.3 mg) ($R_f = 0.48$). Part (118 mg) of fraction A7-15 was purified by preparative TLC (silica gel, *n*-hexane/EtOAc, 2:3) to give **14** (5.3 mg) ($R_f = 0.62$).

4.4.1. 8-Formylalloxanthoxyletin (**1**)

Amorphous powder. UV (MeOH): λ_{\max} (log ϵ) = 224 (4.39), 261 (4.15), 281 (4.11) nm. IR (KBr): $\nu_{\max} = 1729$ (C=O), 1693 (C=O) cm^{-1} . ^1H NMR (CDCl_3 , 500 MHz): $\delta = 1.53$ (6H, s, Me-2' \times 2), 3.92 (3H, s, OMe-7), 5.70 (1H, d, $J = 10.5$ Hz, H-3'), 6.32 (1H, d, $J = 10.0$ Hz, H-3), 6.64 (1H, d, $J = 10.5$ Hz, H-4'), 8.00 (1H, d, $J = 10.0$ Hz, H-4), 10.53 (1H, s, CHO) (Figure S1). ^{13}C NMR (CDCl_3 , 125 MHz): $\delta = 28.5$ (Me-2' \times 2), 63.6 (OMe), 79.7 (C-2'), 105.5 (C-10), 110.7 (C-6), 110.7 (C-8), 113.5 (C-3), 115.4 (C-4'), 129.3 (C-3'), 137.8 (C-4), 155.3 (C-5), 157.5 (C-9), 159.4 (C-2), 160.2 (C-7), 185.7 (CHO) (Figure S2). ESI-MS: $m/z = 309$ $[\text{M} + \text{Na}]^+$ (Figure S3). HR-ESI-MS: $m/z = 309.0739$ $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{16}\text{H}_{14}\text{O}_5\text{Na}$: 309.0739) (Figure S4).

4.4.2. (*E*)-Avicennone (**2**)

Amorphous powder. UV (MeOH): λ_{\max} (log ϵ) = 227 (4.00), 281 (4.16) nm. IR (neat): $\nu_{\max} = 1734$ (C=O), 1695 (C=O) cm^{-1} . ^1H NMR (CDCl_3 , 600 MHz): $\delta = 1.51$ (6H, s, Me-2' \times 2), 2.42 (3H, s, Me-3''), 3.81 (3H, s, OMe-7), 5.70 (1H, d, $J = 10.2$ Hz, H-3'), 6.30 (1H, d, $J = 9.6$ Hz, H-3), 6.59 (1H, d, $J = 10.2$ Hz, H-4'), 7.32 (1H, d, $J = 16.5$ Hz, H-2''), 7.83 (1H, d, $J = 16.5$ Hz, H-1''), 8.02 (1H, d, $J = 9.6$ Hz, H-4) (Figure S5). ^{13}C NMR (CDCl_3 , 150 MHz): $\delta = 28.2$ (Me-2' \times 2), 29.7 (C-4''), 62.4 (OMe), 78.7 (C-2'), 106.3 (C-10), 107.5 (C-8), 110.8 (C-6), 113.2 (C-3), 116.1 (C-4'), 129.5 (C-3'), 130.3 (C-2''), 131.6 (C-1''), 138.2 (C-4), 149.7 (C-5), 154.1 (C-9), 159.2 (C-7), 160.0 (C-2), 199.8 (C-3'') (Figure S6). ESI-MS: $m/z = 349$ $[\text{M} + \text{Na}]^+$ (Figure S7). HR-ESI-MS: $m/z = 349.1056$ $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{19}\text{H}_{18}\text{O}_5\text{Na}$, 349.1052) (Figure S8).

4.4.3. (*Z*)-Avicennone (**3**)

Amorphous powder. UV (MeOH): λ_{\max} (log ϵ) = 226 (4.01), 280 (4.14) nm. IR (KBr): $\nu_{\max} = 1734$ (C=O), 1694 (C=O) cm^{-1} . ^1H NMR (CDCl_3 , 600 MHz): $\delta = 1.49$ (6H, s, Me-2' \times 2), 2.23 (3H, s, Me-3''), 3.72 (3H, s, OMe-7), 5.64 (1H, d, $J = 10.2$ Hz, H-3'), 6.23 (1H, d, $J = 9.6$ Hz, H-3), 6.40 (1H, d, $J = 12.6$ Hz, H-2''), 6.56 (1H, d, $J = 10.2$ Hz, H-4'), 6.79 (1H, d, $J = 12.6$ Hz, H-1''), 7.99 (1H, d, $J = 9.6$ Hz, H-4) (Figure S9). ^{13}C NMR (CDCl_3 , 150 MHz): $\delta = 28.2$ (Me-2' \times 2), 29.7 (C-4''), 62.4 (OMe), 77.8 (C-2'), 105.9 (C-10), 107.9 (C-8), 110.3 (C-6), 113.0 (C-3), 116.2 (C-4'), 128.6 (C-1''), 129.1 (C-3'), 132.3 (C-2''), 138.2 (C-4), 148.9 (C-5), 152.6 (C-9), 156.9 (C-7), 159.8 (C-2), 200.6 (C-3'') (Figure S10). ESI-MS: $m/z = 349$ $[\text{M} + \text{Na}]^+$ (Figure S11). HR-ESI-MS: $m/z = 349.1054$ $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{19}\text{H}_{18}\text{O}_5\text{Na}$, 349.1052) (Figure S12).

4.5. Biological Assay

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

human neutrophils in a concentration-dependent manner. The purity of the tested compounds was >98% as identified by NMR and MS.

4.5.1. 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 [17]. Purified neutrophils containing > 98% viable cells, as determined by the trypan blue exclusion method [18], were re-suspended in a calcium (Ca^{2+})-free HBSS buffer at pH 7.4 and were maintained at 4 °C prior to use.

4.5.2. Measurement of Superoxide Anion Generation

The assay for measurement of superoxide anion generation was based on the SOD-inhibitable reduction of ferricytochrome *c* [19,20]. In brief, after supplementation with 0.5 mg/mL ferricytochrome *c* and 1 mM Ca^{2+} , neutrophils ($6 \times 10^5/\text{mL}$) were equilibrated at 37 °C for 2 min and incubated with different concentrations (10–0.01 $\mu\text{g}/\text{mL}$) of compounds or DMSO (as control) for 5 min. Cells were incubated with cytochalasin B (1 $\mu\text{g}/\text{mL}$) for 3 min prior to the activation with 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* ($\epsilon = 21.1/\text{mM}/10 \text{ mm}$).

4.5.3. Measurement of Elastase Release

Degranulation of azurophilic granules was determined by measuring elastase release as described previously [19,20]. 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 ($6 \times 10^5/\text{mL}$) 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 $\mu\text{g}/\text{mL}$), 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.

4.5.4. Statistical Analysis

Results are expressed as the mean \pm standard error of the mean (SEM), and comparisons were made using Student's *t*-test. A probability of 0.05 or less was considered significant. The software SigmaPlot was used for the statistical analysis.

5. Conclusions

Eighteen compounds, including three new coumarins (**1–3**), were isolated from the stem bark of *Z. avicennae*. The structures of these compounds were established on the basis of spectroscopic data. Reactive oxygen species (ROS) (e.g., superoxide anion ($\text{O}_2^{\bullet-}$), hydrogen peroxide) and granule proteases

(e.g., elastase, cathepsin G) produced by human neutrophils contribute to the pathogenesis of inflammatory diseases. The effects on neutrophil pro-inflammatory responses of isolates were evaluated by suppressing fMLP/CB-induced $O_2^{\cdot-}$ generation and elastase release by human neutrophils. The results of anti-inflammatory experiments indicate that compounds **1**, **2**, **4**, **9**, **11**, and **15** can significantly inhibit fMLP-induced $O_2^{\cdot-}$ generation and/or elastase release. 8-Formylalloxanthoxyletin (**1**), alloxanthoxyletin (**4**), and xanthoxyletin (**9**) were the most effective among the isolated compounds, with IC_{50} values of $4.60 \pm 0.83/2.59 \pm 0.52$, $1.47 \pm 0.53/3.43 \pm 0.63$, and $1.47 \pm 0.41/4.18 \pm 0.73$ $\mu\text{g/mL}$, respectively, against fMLP-induced $O_2^{\cdot-}$ generation and elastase release. Our study suggests *Z. avicennae* and its isolates (especially **1**, **4**, and **9**) are worthy of further biomedical investigation and could be expectantly developed as potential candidates for the treatment or prevention of various inflammatory diseases.

Supplementary Materials

Supplementary materials can be found at <http://www.mdpi.com/1422-0067/16/05/9719/s1>.

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Author Contributions

Jih-Jung Chen designed the research; Chieh-Kai Yang, Yueh-Hsiung Kuo, Tsong-Long Hwang, and Wen-Lung Kuo performed the experiments; Jih-Jung Chen, Chieh-Kai Yang, Yun-Ping Lim, Ping-Jyun Sung, Tsung-Hsien Chang, and Ming-Jen Cheng contributed to the analysis of the data; Jih-Jung Chen wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

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