(–)-Xanthienopyran, a New Inhibitor of Superoxide Anion Generation by Activated Neutrophils, and Further Constituents of the Seeds of *Xanthium strumarium*

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Key words
- *Xanthium strumarium*
- Asteraceae
- Cang-Er Zi
- superoxide anion generation inhibitor
- neutrophils

Abstract

The dried seeds of *Xanthium strumarium* (Asteraceae) are used after thorough stir-frying as an ingredient in traditional Chinese medicines for relieving allergy. Two new compounds, xanthialdehyde (**2**) and (–)-xanthienopyran (**7**), as well as 26 known compounds were isolated in the present study. The structures of the isolates were elucidated by spectroscopic methods. Among them, compound **7** exhibited significant selective inhibition of superoxide anion generation by human neutrophils induced by formyl-L-methionyl-L-leucyl-L-phenylalanine, with an IC_{50} value of 1.72 μg/mL.

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Introduction

Neutrophils are known to play an important role in host defense against invasion by microorganisms and in the pathogenesis of various diseases such as rheumatoid arthritis, ischemia-reperfusion injury, chronic obstructive pulmonary disease, and asthma [1]. In response to diverse stimuli, activated neutrophils secrete a series of cytotoxins, such as the superoxide anion, a precursor of other reactive oxygen species, and granule proteases. Suppression of extensive or inappropriate activation of neutrophils using drugs has been proposed as a way to ameliorate these inflammatory diseases [1], [2], [3]. However, there are only a few agents currently available that directly modulate neutrophil proinflammatory responses in clinical practice. Therefore, the research and development of new-generation anti-inflammatory drugs is an important issue.

Plants of the Asteraceae family are important sources of food and medicinal materials in Asia. Extracts of these plants are used to treat diseases in combination with other herbs in many traditional Chinese medicines [4]. One of these, Cang-Er Zi, is made from the seeds of *Xanthium strumarium* L. (also called *X. sibiricun* Patrin ex Wider and *X. strumarium* L. var. *japonicum*), which is distributed along river banks and seacoasts throughout lowlands in Taiwan. This plant has been used to treat some allergic conditions, arthritis, common colds, headache, and hypoglycemia. In recent studies, extracts of this plant exhibited anti-inflammatory, analgesic, and antinociceptive effects [5], [6]. In continuing research on the development of anti-inflammatory agents from natural sources, we isolated 28 compounds. Among them, **2** and **7** are new (see **Fig. 1**). Compounds **9**–**16**, **27**, and **28** were isolated from the genus *Xanthium* for the first time. In addition, compounds **1**, **3**, **7**, **8**, **11**, **14**, **20**, a mixture of **23** and **24** (3 : 1), and a mixture of **25** and **26** (4 : 1) showed weak cytotoxicity against the HONE-1 (human nasopharyngeal carcinoma) and NUGC-3 (human gastric cancer) cell lines. Furthermore, compound **7** exhibited significant selective inhibition of superoxide anion generation by human neutrophils induced by formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), with an IC_{50} value of 1.72 μg/mL. The isolation, structural elucidation, and cytotoxicity data for these new compounds are reported here.

Materials and Methods

General experimental procedures

Optical rotations were determined with a JASCO DIP-1000 digital polarimeter (cell length 10 mm). UV and IR spectra were measured on a Hitachi 200–20 and a Mattson Genesis II spectrophotometer, respectively. NMR spectra were recorded on Varian Gemini 2000 300 MHz NMR spectrometers with TMS as internal standard.

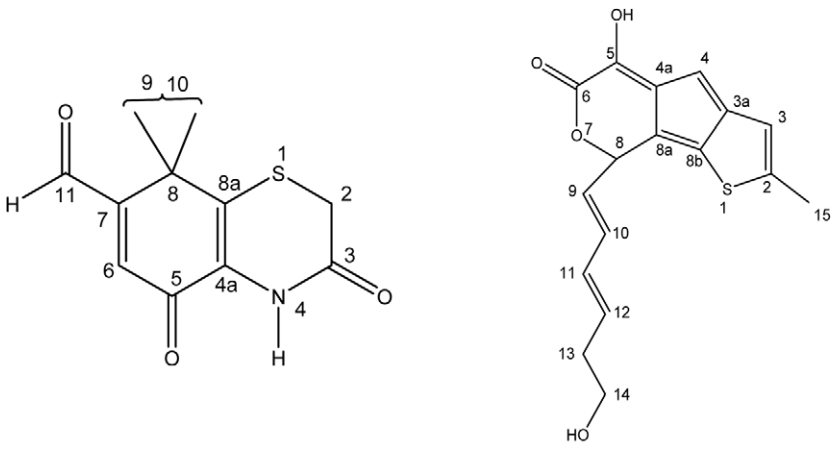

Fig. 1 Structures of the new isolates.

LR-FAB-MS and LR-EI-MS were measured on a JEOL JMS-SX/SX 102A mass spectrometer or a Quattro GC/MS spectrometer. Silica gel 60 (Macherey-Nagel; 230 – 400 mesh) was used for column chromatography. TLC was performed on silica gel (Macherey-Nagel; SIL G-25 UV_{254}, 0.25 mm). The HPLC systems 1 (Develosil C30-UG-5 20 × 250 mm MeCN/H_2O 40/60, flow rate 3 mL/min) and 2 (Hypersil ODS 5μ 4.6 × 250 mm MeCN/H_2O 50/50, flow rate 1 mL/min) were used under isolation procedures. The spots were detected by spraying with Dragendorff's reagent or 50% H_2SO_4 followed by heating on a hot plate.

Plant material

The fresh seeds of *X. strumarium* L. were purchased from the Chinese herb shop in Kaohsiung City, Taiwan, in July 2000. Voucher specimens (Xanthium01) were identified by Dr. Ming-Hon Yen and deposited in the Graduate Institute of Natural Products, Kaohsiung, Taiwan.

Extraction and isolation

The fresh seeds of *X. strumarium* (10 kg) were purchased from Kaohsiung City and extracted with methanol (25 L × 6). The methanolic extracts (1630 g) were partitioned between *n*-hexane (2 L × 5), $CHCl_3$ (2 L × 5), and *n*-BuOH (2 L × 5) with water (2 L) to yield *n*-hexane (1050 g), $CHCl_3$ (50 g), *n*-BuOH (300 g), and H_2O layers, respectively. The *n*-hexane layer was further partitioned with methanol to yield *n*-hexane (800 g) and methanol (250 g) layers. The $CHCl_3$ layer (50 g) was separated by silica gel (3.0 kg) column chromatography with a stepwise gradient of $CHCl_3$-MeOH (100 : 1, 50 : 1, 20 : 1, 10 : 1, 5 : 1, and 4 : 1, each 2 L) to afford 9 fractions (C-1–C-9). Compounds **2** (3.2 mg, *n*-hexane/acetone 3 : 1, R_f = 0.35), **4** (1.1 mg, system 1, t_R = 79.4 min), **5** (2.2 mg, system 1, t_R = 82.6 min), **6** (1.2 mg, *n*-hexane/EtOAc 10 : 1, R_f = 0.25), **9** (3.5 mg, *n*-hexane/EtOAc 1 : 2, R_f = 0.25), **19** (0.5 mg, *n*-hexane/EtOAc 3 : 1, R_f = 0.5), and **20** (3.8 mg, *n*-hexane/EtOAc 10 : 1, R_f = 0.5) were isolated from fraction C-1 by reversed-phase HPLC. Compounds **11** (13.0 mg, *n*-hexane/EtOAc 1 : 1, R_f = 0.5), **24** (17.9 mg, $CHCl_3$/methanol 15 : 1, R_f = 0.5), and **21** (4.0 mg, $CHCl_3$/methanol 10 : 1, R_f = 0.91) were collected from fraction C-2 by silica gel column chromatography (20.0 g). Compounds **1** (460.5 mg, *n*-hexane/EtOAc 1 : 4, R_f = 0.5), **7** (15.1 mg, pure EtOAc, R_f = 0.5), **10** (9.2 mg, *n*-hexane/EtOAc 1 : 4, R_f = 0.5), **12** (7.2 mg, *n*-hexane/EtOAc 1 : 1, R_f = 0.45), **18** (9.3 mg, *n*-hexane/EtOAc 1 : 1, R_f = 0.5), and the mixture of **25** and **26** (1936.0 mg, $CHCl_3$/methanol 20 : 1, R_f = 0.5) were isolated from fraction C-3 by repeated column chromatography and by preparative TLC. Compounds **23** and **24** were purified and obtained from the methanol layer by column chromatography and recrystallization, respectively. Additionally, the *n*-BuOH layer (300 g) was separated into 9 fractions (B-1–B-9) by column chromatography on silica gel (4 kg) with a stepwise gradient of EtOAc-MeOH (100 : 1, 50 : 1, 20 : 1, 10 : 1, and 5 : 1, each 3 L). Fraction B-2 was separated by repeated column chromatography to yield **8** (135.6 mg, system 2, flow rate 1 mL/min, t_R = 18.7 min) and **17** (4.0 mg, $CHCl_3$/methanol 4 : 1, R_f = 0.4). Compounds **3** (63.7 mg, system 2, t_R = 21.04 min), **13** (1.0 mg, system 2, t_R = 98.0 min), **14** (1.0 mg, system 2, t_R = 85.18 min), **15** (0.8 mg, system 2, t_R = 149.3 min), **16** (0.4 mg, system 2, t_R = 210.7 min), and **22** (0.9 mg, system 2, t_R = 29.1 min) were isolated and purified from fraction B-3 by Diaion HP-20 and silica column chromatography. Compound **28** (4.8 mg, *n*-hexane/EtOAc 10 : 1, *Rf* = 0.50) was isolated from fraction B-6 by column chromatography with gradient mixtures of an EtOAc-MeOH system.

Isolates

Xanthialdehyde (**2**): Yellow needles; m. p. 156 – 158 °C; UV (MeOH): ν_{max} = 383, 300, 251 nm; IR (neat): ν_{max} = 3208, 3118, 1685, 1650, 1587 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$) and ^{13}C-NMR (100 MHz, $CDCl_3$), see **Table 1**. FAB-MS: *m*/*z* (rel. int.) = 238 $[M+H]^+$ (15), 237 $[M]^+$ (12), 154 (100), 137 (94), 136 (98), 77 (55); HR-FAB-MS: *m*/*z* = 237.0465 $[M]^+$ (calcd. for $C_{11}H_{11}NO_3S$: 237.0460).

(–)-Xanthienopyran (**7**): Yellow powder; m. p. 218 – 220 °C; $[\alpha]_D^{25}$: –27.9 (*c* 0.47, CH_3OH); UV (MeOH): ν_{max} = 331, 254, 226 nm; IR (neat): ν_{max} = 3298, 2919, 2855, 1739, 1621, 1590, 1427 cm^{-1}; ^{1}H-NMR (200 MHz, CD_3COCD_3) and ^{13}C-NMR (50 MHz, CD_3COCD_3), see **Table 2**; FAB-MS: *m*/*z* (rel. int.) = 317 $[M+H]^+$ (14), 316 $[M]^+$ (7), 219 (26), 192 (92), 176 (62), 149 (62); molecular formula: $C_{17}H_{16}O_4S$.

Bioassays

All isolates from the seeds of *X. strumarium* were purified for use in bioassays (purity > 99%).

Neutrophil O_2^- generation: This assay was carried out according to established protocols [11].

Table 1 ^{1}H- and ^{13}C-NMR ($CDCl_3$) spectral data of xanthialdehyde (**2**)

Position	2		
	δ_H (mult; J in Hz)	δ_C	HMBC ($^{1}H \rightarrow ^{13}C$)
2	3.48 (2H, *s*)	29.0	C-3, C-8a
3		160.9	
4	8.16 (1H, *s*)		
4a		129.7	
5		174.5	
6	6.96 (1H, *s*)	139.3	C-4a, C-7, C-8, C-11
7		158.6	
8		41.4	
8a		144.6	
9	1.60 (6H, *s*)	26.2	C-7, C-8, C-8a
10	1.60 (6H, *s*)	26.2	C-7, C-8, C-8a
11	9.69 (1H, *s*)	191.9	C-8

Cytotoxicity assays: Fractions and isolates were tested against HONE-1 (human nasopharyngeal carcinoma) and NUGC-3 (human gastric cancer) cancer cell lines with an ELISA reader. Cancer cells were seeded at 5 × 10 cells/mL into 24-well plates and then incubated for 24 h at 37 °C in a 5 % CO_2 incubator. After 24 hours, the drugs were added, incubation was continued for 72 hours, and 1 % methylene blue was added to stain the proteins for 40 min. Then 1 % sarcosyl was added to wells for 24 h after water washing 2 – 3 times and drying. The optical density (O.D.) was measured at 590 nm.

Results and Discussion

Bioassay-directed fractionation of *X. strumarium* seeds yielded 28 compounds, including: three thiazines, xanthiazone (**1**) [7], xanthialdehyde (**2**), and xanthiazone-*O*-*β*-D-glucoside (**3**) [4]; three xanthanolides, xanthinosin (**4**) [8], 11*β*,13-dihydroxanthinosin (**5**), and xandiversifolide (**6**); one thienocyclopyran, (–)-xanthienopyran (**7**) [9], [10]; one lactone, (2*S*,3*R*)-2,3-dihydroxy-2-methylbutyrolactone (**8**); one lignan, (–)-pinoresinol (**9**); one neolignan, (7*S*,8*R*)-(+)-dihydrodehydroconiferyl alcohol (**10**); two indoles, indole-3-carboxaldehyde (**11**) and indole-3-carboxylic acid (**12**); 10 benzenoids, caffeic acid (**17**), methyl caffeate (**18**), coniferaldehyde (**19**), vanillin (**20**), vanillic acid (**21**), 2,4′-dihydroxydiphenyl methane (**13**), 4,4′-dihydroxydiphenyl methane (**14**), 3-methyl-4,4′-dihydroxydiphenyl methane (**15**), 2,4-bis(4-hydroxybenzyl)phenol (**16**), and *p*-hydroxybenzaldehyde (**22**); one imide, succinimide (**27**); one furan, 5-(hydroxymethyl)furfural (**28**); and four steroids, *β*-sitosterol (**23**), stigmasterol (**24**), *β*-sitosteryl-*β*-D-glucoside (**25**), and stigmasteryl-*β*-D-glucoside (**26**). Among these, xanthialdehyde (**2**) and (–)-xanthienopyran (**7**) are new compounds isolated from the $CHCl_3$ layer.

Compound **2** was obtained as yellow needles. HR-FAB-MS of **2** showed an $[M]^+$ ion at m/z = 237.0465 ($C_{11}H_{11}NO_3S$), for which we deduced seven degrees of unsaturation. The IR spectrum showed absorption peaks for NH (3208 cm^{-1}) and carbonyl (1685 cm^{-1}) groups. 1 D NMR data (**Table 1**) revealed 11 carbon signals, including two methyls, one methylene, two sp^2 methines, one sp^3 quaternary carbon, and five sp^2 quaternary carbons. Among the five sp^2 quaternary carbons, two should be carbonyl carbons according to their chemical shifts at δ = 160.9 and 174.5. 1 D NMR and HMQC data indicated the presence of two methyl groups at δ_H = 1.60 (6H, s, δ_C = 26.2), one methylene group at δ_H 3.48 (2H, s, δ_C = 29.0), one olefinic proton at δ_H = 6.96 (1H, s, δ_C = 139.3), and an aldehyde group at δ_H 9.69 (1H, s, δ_C = 191.9). The key HMBC connections are shown in **Table 1**. Based on the above spectral data, the skeleton of **2** is similar to that of xanthiazone (**1**) [4]. The only difference is an aldehyde group (δ_H = 9.69, 1H, s, δ_C = 191.9) at C-7 in **2** instead of an oxymethylene group (δ_H = 4.65, 2H, *d*, *J* = 1.8 Hz, δ_C = 60.4) in **1** according to the aldehyde group exhibiting J_3 interaction, with δ_C = 139.3 (C-6), and J_2 interaction, with δ_C = 158.6 (C-7), rather than the oxymethylene group exhibiting J_3 interaction, with δ_C = 121.2 (C-6), and J_2 interaction, with δ_C = 170.3 (C-7). Thus, the new compound was named xanthialdehyde (**2**).

Compound **7** was obtained as an orange powder. FAB-MS of **7** revealed an $[M+H]^+$ ion at m/z = 317. The UV spectrum had three absorption peaks at 331 nm, 254 nm, and 226 nm. The IR absorption peaks at 3298 cm^{-1} and 1739 cm^{-1} supported the presence of hydroxyl and carbonyl groups, respectively. ^{13}C and DEPT

Table 2 ^{1}H- and ^{13}C-NMR (CD_3COCD_3) spectral data of (–)-xanthienopyran (**7**)

Position	δ_C	δ_H (mult; J in Hz)	HMBC ($^{1}H \rightarrow ^{13}C$)	NOESY
2	145.2			
3	122.4	7.09 (1H, *d*, *J* = 1.0 Hz)	C-2, C-3a, C-8b	
3a	145.6			
4	115.0	7.50 (1H, *s*)	C-3, C-5, C-8a, C-8b	
4a	122.0			
5	151.6			
6	170.6			
8	82.2	6.09 (1H, *d*, *J* = 6.8 Hz)		H-9
8a	135.9			
8b	127.7			
9	126.7	5.74 (1H, *dd*, *J* = 15.2, 6.8 Hz)		H-8, H-10
10	135.8	6.52 (1H, *dd*, *J* = 15.2, 10.4 Hz)		H-9, H-11
11	132.2	6.17 (1H, *dd*, *J* = 15.4, 10.4 Hz)		H-10, H-12
12	135.5	5.86 (1H, *dt*, *J* = 15.4, 7.0 Hz)		H-11, H-13
13	37.8	2.30 (2H, *q*, *J* = 6.8 Hz)		H-12, H-14
14	62.7	3.59 (2H, *t*, *J* = 6.8 Hz)		H-13
15	16.7	2.60 (3H, *d*, *J* = 1.0 Hz)	C-2, C-3	H-3

		Compounds	
		Xanthiazone (1)	(–)-Xanthienopyran (7)
fMLP inhibitory concentration (μg/mL)	10.00	6.49 ± 0.78	91.42 ± 0.51
	3.00	N	70.59 ± 4.23
	1.00	N	35.77 ± 8.52
IC_{50}		N	1.72 ± 0.62

Table 3 Inhibitory effects (%) of compounds from the seeds of *X. strumarium* L. on superoxide anion generation by human neutrophils in response to fMLP

Notes: n = 3 – 4; N = not determined; results are mean ± SEM. Diphenyleneiodonium was used as positive control in the anti-inflammatory assay. It exhibited significantly (with an IC_{50} value of 0.16 μg/mL) selective inhibition of superoxide anion generation by human neutrophils that were stimulated by fMLP/CB.

data indicated the presence of one methyl, two methylene, one sp^3 methine, six sp^2 methine, and seven sp^2 quaternary carbons (**Table 2**). 1 D NMR and HMQC data indicated the presence of one methyl group at $\delta_H = 2.60$ (3H, d, $J = 1.0$ Hz, $\delta_C = 16.7$), two methylene groups at $\delta_H = 2.30$ (2H, q, $J = 6.8$ Hz, $\delta_C = 37.8$) and 3.59 (2H, t, $J = 6.8$ Hz, $\delta_C = 62.7$), and two sp^2 methine carbons at $\delta_H = 7.09$ (1H, d, $J = 1.0$ Hz, $\delta_C = 122.4$) and 7.50 (1H, s, $\delta_C = 115.0$). In addition, the other sp^2 methine carbons at $\delta_H = 6.52$ (1H, dd, $J = 15.2$, 10.4 Hz), 6.17 (1H, dd, $J = 15.4$, 10.4 Hz), 5.86 (1H, dt, $J = 15.4$, 7.0 Hz), and 5.74 (1H, dd, $J = 15.2$, 6.8 Hz) indicated the presence of conjugated double bonds, which was confirmed by the COSY spectrum. The key HMBC and NOESY correlations are listed in **Table 2**. Comparison with literature data [8] revealed that the structure of **7** is almost the same as that of xanthienopyran. The only difference is the positive rotation index ([α]: + 7.91) for (+)-xanthienopyran, whereas compound **7** has a negative rotation index ([α]: –27.9). Therefore, compound **7** was identified as a new compound named (–)-xanthienopyran.

All compounds were screened for anti-inflammatory activity and cytotoxicity against two human cancer cell lines, human nasopharyngeal carcinoma (HONE-1) and gastric carcinoma (NUGC-3). All isolates exhibited very weak inhibitory activity towards the cancer cells at concentrations of 10 μM and 50 μM. In recent studies, extracts of this plant exhibited potent inhibition of NO, PGE_2, and TNF-α production [5], [6]. In the present study, (–)-xanthienopyran (**7**) isolated from *X. strumarium* exhibited selective inhibition of O_2^- generation by human neutrophils induced by fMLP, with an IC_{50} value of 1.72 ± 0.62 μg/mL (**Table 3**). Because **7** did not inhibit PMA-induced superoxide anion generation in human neutrophils, it may inhibit signaling upstream of protein kinase C [1].

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