รายงานวิจัยฉบับสมบูรณ์

เรื่อง

การดัดแปลงโครงสร้างสารกลุ่มแซนโทนต้านเชื้อแบคทีเรีย เพื่อฤทธิ์ที่ดีกว่าหรือความเป็นพิษลดลง

A Better Activity or Less Toxic: Structure Modification of Antibacterial Xanthones

โดย

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โครงการวิจัยนี้ได้รับทุนสนับสนุนการวิจัยจาก เงินรายได้มหาวิทยาลัย

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Contents

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บทคัดย่อ

จากผลการศึกษาการเพิ่มฤทธิ์การยับยั้งเชื้อแบคทีเรียและการลดความเป็นพิษของสารประกอบกลุ่มแซนโทน พบว่าสารประกอบ **5a** แสดงฤทธิ์ยับยั้งเชื้อแบคทีเรียชนิด MRSA และ *B. substilis* ได้ดีมากด้วยค่า MIC เท่ากับ 2.34 μg/mL เทียบเท่ากับสารตั้งต้น แต่โดยส่วนใหญ่สารที่มีการดัดแปลงโครงสร้างแสดงฤทธิ์การต้านเชื้อแบคทีเรียน้อยกว่าเมื่อเทียบกับสารตั้งต้น นอกจากนี้ได้ขยายขอบเขตงานวิจัยโดยการศึกษาการทดสอบฤทธิ์การยับยั้งอนุมูลอิสระชนิดไนตริกออกไซด์ (nitric oxide) ที่ถูกสร้างมาจากเซลล์ชนิด RAW264.7 ของสารสกัดและสารบริสุทธิ์ที่แยกได้จากรากของต้นติ้วขน การแยกสารบริสุทธิ์จากส่วนสกัดหยาบไดคลอโรมีเทน (crude CH_2Cl_2 extract) จากรากของต้นติ้วขนสามารถแยกสารบริสุทธิ์ในกลุ่มของ *tri*-oxygenated xanthone ได้ทั้งหมด 6 สาร คือสารประกอบ **1**-**6** และสารบริสุทธิ์ในกลุ่มของ *tetra*-oxygenated xanthone ได้อีกทั้งหมด 4 สาร คือสารประกอบ **7**-**10** ตามลำดับ สารประกอบ **7** แสดงฤทธิ์ยับยั้งการหลั่งอนุมูลอิสระชนิดไนตริกออกไซด์ด้วยค่า IC_{50} = 3.9 μM และสารประกอบ **8** แสดงฤทธิ์ยับยั้งการหลั่งอนุมูลอิสระชนิดไนตริกออกไซด์รองลงมาด้วยค่า IC_{50} = 4.3 μM ตามลำดับ เพื่อให้เข้าใจในกลไกการต้านการอักเสบของสารประกอบ **7** และ **8** จึงได้มีการศึกษาผลการแสดงออกของ *m*-RNA ยีน (gene expression) 2 ชนิด คือ *i*NOS (inducible nitric oxide syntheses) และ COX-2 (cyclooxygenase 2) ที่เกี่ยวข้องกับกลไกการอักเสบ พบว่าสารประกอบ **7** สามารถลดการแสดงออกของยีน *i*NOS และ COX-2 ได้ทั้งคู่ โดยที่ขึ้นอยู่กับค่าความเข้มข้น (dose-dependent) ของสารประกอบ **7** ด้วย ส่วนสารประกอบ **8** สามารถลดการแสดงออกของยีน *i*NOS ได้อย่างเดียว โดยไม่มีผลต่อการแสดงออก COX-2 ซึ่งสามารถสรุปได้ว่าสารประกอบกลุ่มแซนโทนที่อยู่ในส่วนสกัดหยาบจากรากต้นติ้วขนน่าจะเป็นสารประกอบหลักที่แสดงฤทธิ์ต้านการอักเสบ

Abstract

The enhancing anti-bacterial activity and reducing toxicity by structures modification in this study revealed that most of the modified compounds exhibit anti-bacterial activity less than their precursors, except compound **5a** showed strong activity against MRSA and *B. substilis* with MIC value of 2.34 μg/mL comparative to those of α-mangostin. Moreover, the study was extended to investigate the inhibitory activity of the extract and isolated compounds from the roots of *Cratoxylum formosum* ssp. *pruniflorum*. Isolation of the CH_2Cl_2 extract of *C. formosum* ssp. *pruniflorum* roots afforded ten known xanthones including six *tri*-oxygenated xanthones and four *tetra*-oxygenated xanthones, respectively. Compound **7** showed the highest inhibitory activity against NO release with an IC_{50} value of 3.9 μM, followed by compound **8** with an IC_{50} value of 4.3 μM, respectively. In order to understand the mechanism of this anti-inflammatory activity, the transcriptional level of compound **7** was found to down regulate mRNA expressions of iNOS and COX-2 in dose-dependent manners, whereas compound **8** inhibited only iNOS mRNA expression but did not affect on that of COX-2 gene. Xanthones might be the main anti-inflammatory components in *C. formosum* ssp. *pruniflorum*.

Executive Summary

Introduction

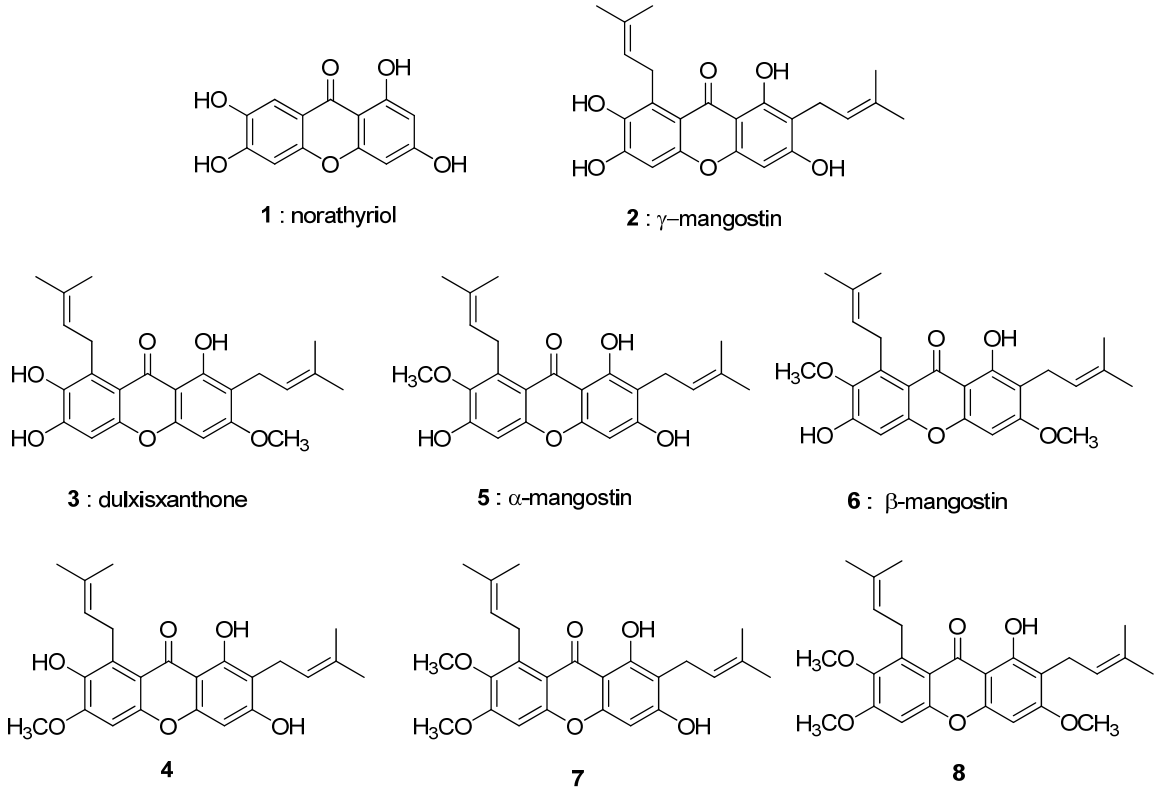
Considerable attentions have been devoted to biological compounds. It is well documented that bacterial infection is becoming serious problems of human being. Many herbs have demonstrated anti-bacterial activity. *Cratoxylum formosum* ssp. *pruniflorum* is a shrub tree belonging to the Guttiferae family (Smitinand, 2001). It is widely distributed around the Southeast Asia particularly around the northern zone of Southeast Asian such as Laos, Cambodia and Thailand, known as "Tui-Khon" in Thailand. Most importantly, some of isolated xanthones from *Cratoxylum* plants have been found to possess potent anti-bacterial activity. In the present study is aimed to search for potent natural and modified bacterial agents for the prevention and treatment of bacterial infection.

Objectives

- To extract and isolate xanthones from the Hulks of *G. mangostana* and the roots of *C. formosum* ssp. *pruniflorum* and to modified an isolated xanthone for the enhancing anti-bacterial activity and reducing their toxicity factors
- To study the influent of substituent group on anti-bacterial activity
- To extract and isolate xanthones from the roots of *C. formosum* ssp. *pruniflorum* for the anti-inflammatory activity

Result and Discussions

The crude CH_2Cl_2 extracts of the roots of *Cratoxylum formosum* ssp. *pruniflorum* and the hulks of *Garcinia mangostana* were subjected to chemical investigation leading to the isolation of five known 1,3,6,7-*tetra*-oxygenated xanthones identified as norathyriol (**1**) (Noro et al., 1984; Don et al., 2004), γ-mangostin (**2**) (Mahabusarakam & Wiriyachitra 1987), dulxisxanthone F (**3**) (Dechathai *et al*., 2005), α-mangostin (**5**) (Mahabusarakam *et al*., 1987) and β-mangostin (**6**) (Mahabusarakam *et al*., 1987). Their structures were elucidated by NMR analysis and comparison of their spectroscopic data with those reported in the literatures.



Scheme 1. The structures of compounds **1-8**

Compounds **1-8** have been evaluated for their antibacterial activity against both Gram-(+) and Gram-(-) bacteria. From the anti-bacterial activity result as shown in **Table 1**, the result showed that norathyriol (**1**) was to be inactive against both Gram-(+) and Gram-(-) bacteria, whereas γ-mangostin (**2**), which substituted with two isoprenyl side chains, showed better anti-bacterial activity than norathyriol (**1**), which could be suggested that the bearing of isoprenyl side chain on the *tetra*-oxygenated xanthones nucleus can increase the anti-bacterial activity compared to norathyriol (**1**) and compound **6a**. The *α*-mangostin (**5**) showed strong activity against MRSA, *B substilis* and *P. aureginosa* with MIC value of 2.34 μg/mL, whereas *β*-mangostin (**6**) showed moderate activity against MRSA and *P. aureginosa* with MIC values of 4.68 and 9.37 μg/mL, respectively. It could be suggested that the attachment of methoxyl group affect to its activity.

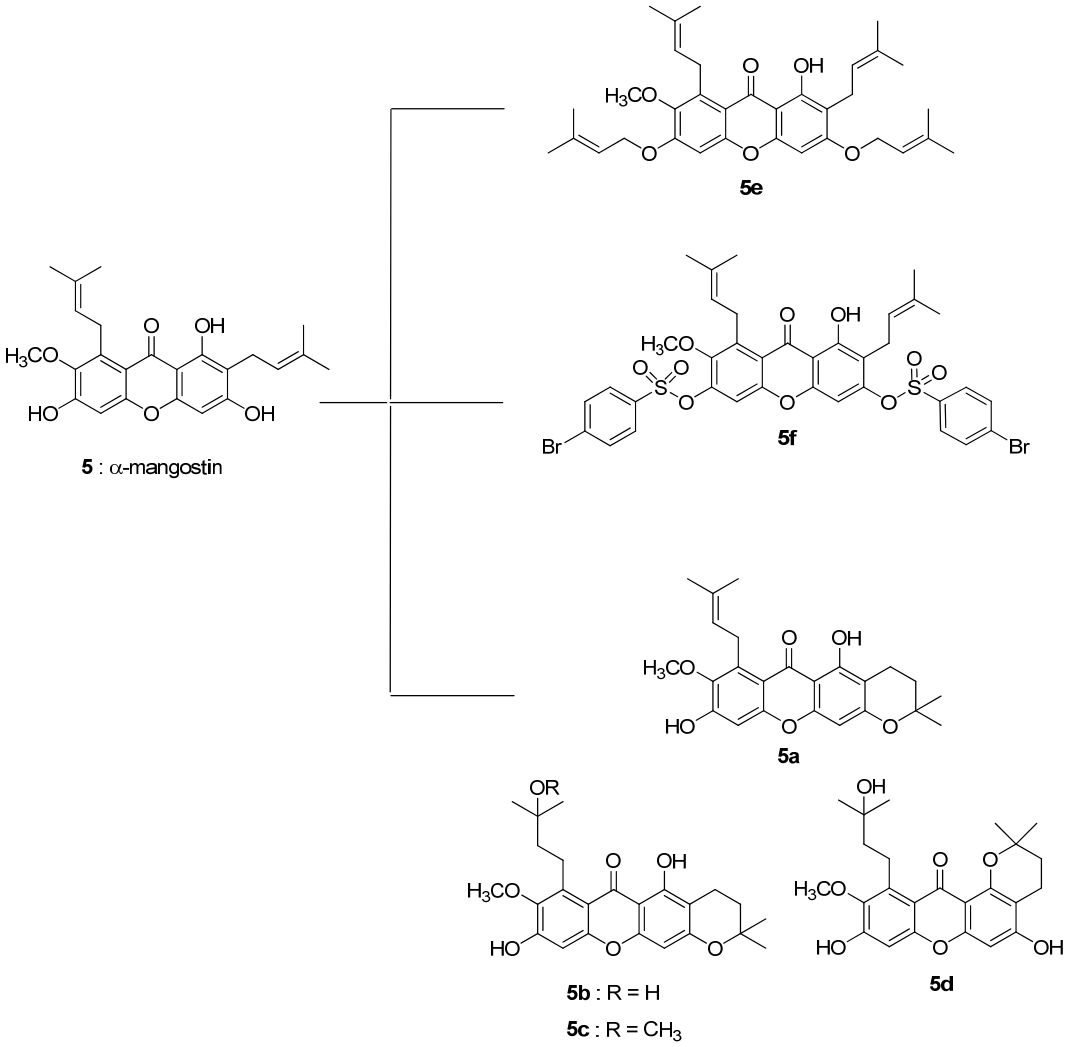
Table 1. Antimicrobial activity (MIC, μg/mL) of **1-8**

Compounds	Antibacterial activity								Antifungal activity
	Gram-positive bacteria [a]					Gram-negative bacteria [b]			
	MRSA	*S. aureus*	*B. subtilis*	*E. faecalis*	VRE	*S. typhi*	*S. sonei*	*P. aeruginosa*	*C. albicans* [c]
1	18.75	150	37.5	300	>300	>300	>300	37.5	>300
2	4.68	18.75	18.75	9.37	NT	18.75	18.75	18.75	NT
3	9.37	18.75	18.75	300	300	150	150	18.75	>300
5	2.34	300	2.34	150	150	18.75	150	2.34	150
6	4.68	300	2.34	300	300	>300	>300	9.37	300
4	9.37	75	75	75	NT	37.5	75	4.67	NT
7	18.75	150	75	75	NT	150	150	18.75	NT
8	>300	300	75	150	300	300	>300	>300	>300
vancomycin	<2.34	<2.34	<2.34	<2.34	<2.34	<2.34	<2.34	<2.34	<2.34

[a] *Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus faecalis* TISTR 459, Methicillin-Resistant *Staphylococcus aureus* (MRSA) ATCC 43300, Vancomycin-Resistant *Enterococcus faecalis* (VRE) ATCC 51299. [b] *Salmonella typhi*, *Shigella sonei* and *Pseudomonas aeruginosa*. [c] *Candida albicans*

To proof this hypothesis, the methylated of γ-mangostin was prepared by treatment with MeI/K_2CO_3 in methanol to give compounds **4**, **7** and **8**, which were tested for their anti-bacterial activity. The result showed that compounds **4**, **7** and **8** were to be inactive than its precursor. It could be suggested that 1,3,6,7-*tetra*-oxygenated-2,8-diprenylated xanthones bearing a methoxyl group at C-7 was the most effective for anti-bacterial agent

From the anti-bacteirlal activity in **Table 1**, it was suggested that α-mangostin (**5**) and β-mangostin (**6**) were the best candidate for anti-bacterial agent. For the next, we attempt to modify structures of α-mangostin (**5**) and β-mangostin (**6**) to reducing their toxicity factors and also to improve or maintain their bioactivity. Installation of isoprenyl and *p*-bromobenzenesulfonyl side chains at C-3 and C-7 of α-mangostin afforded compounds **5e** (Ha *et al*., 2009) and **5f**.



Scheme 2. Synthesis of α-mangostin analogs **5a**-**5f**

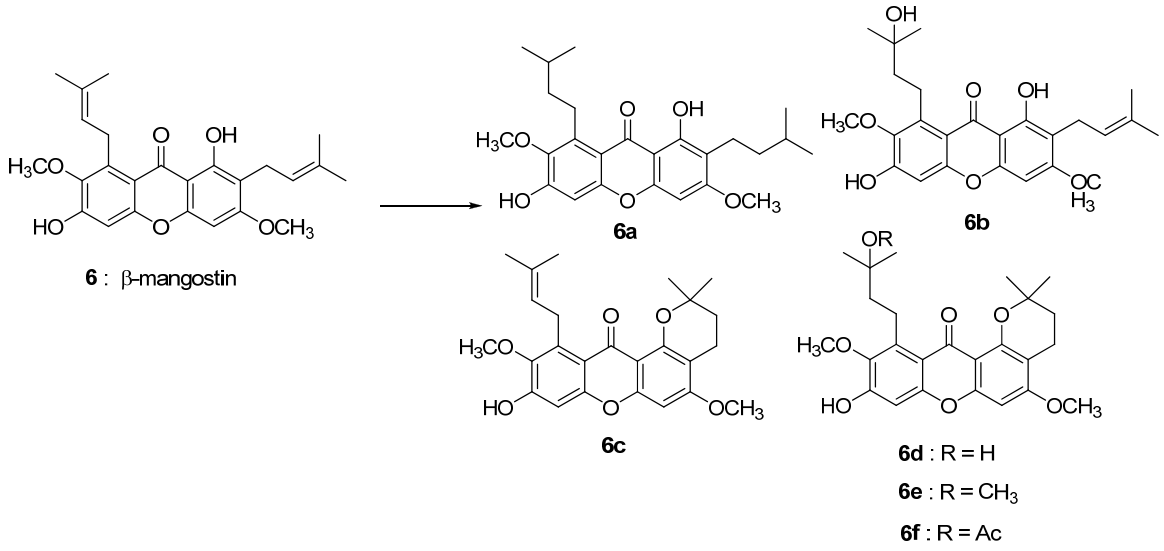
Table 2. Antimicrobial activity (MIC, μg/mL) of **5a-5f** and **6a-6f**

Compounds	Antibacterial activity								Antifungal activity
	Gram-positive bacteria [a)]					Gram-negative bacteria [b)]			
	MRSA	*S. aureus*	*B. subtilis*	*E. faecalis*	VRE	*S. typhi*	*S. sonei*	*P. aeruginosa*	*C. albicans* [c]
5	2.34	300	2.34	150	150	18.75	150	2.34	150
5a	2.34	300	2.34	>300	>300	300	300	9.37	300
5b	18.75	300	18.75	>300	>300	150	300	75	>300
5c	9.37	300	9.37	>300	>300	300	300	75	>300
5d	37.5	300	37.5	>300	>300	300	300	300	>300
5e	>300	>300	>300	300	NT	>300	>300	>300	NT
5f	>300	>300	>300	>300	NT	>300	>300	>300	NT
6	4.68	300	2.34	300	300	>300	>300	9.37	300
6a	37.5	150	150	75	NT	150	150	150	NT
6b	18.75	300	9.37	150	>300	300	300	18.75	>300
6c	>300	300	75	75	150	300	300	300	300
6d	150	300	150	>300	>300	300	300	300	300
6e	>300	150	300	>300	>300	150	300	>300	>300
6f	>300	75	300	>300	>300	300	300	>300	>300
vancomycin	<2.34	<2.34	<2.34	<2.34	<2.34	<2.34	<2.34	<2.34	<2.34

[a)] *Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus faecalis* TISTR 459, Methicillin-Resistant *Staphylococcus aureus* (MRSA) ATCC 43300, Vancomycin-Resistant *Enterococcus faecalis* (VRE) ATCC 51299. [b)] *Salmonella typhi*, *Shigella sonei* and *Pseudomonas aeruginosa*. [c)] *Candida albicans*

Installation of isoprenyl and *p*-bromobenzenesulfonyl side chains at C-3 and C-7 of α-mangostin afforded compounds **5e** and **5f**. The anti-bacterial activity result (**Table 2**) showed that they were inactive. Now, we have to increase its hydrophilicity by installation the hydrophilic part to their isoprenyl side chain by reacting of α-mangostin with acidic medium to yield compounds **5a**-**5d**. Then, only compound **5a** showed strong activity against MRSA and *B. substilis* with MIC value of 2.34 μg/mL comparative to those α-mangostin.

For the structural modification of β-mangostin, treatment of β-mangostin with acidic medium to yield compounds **6b**-**6f**.

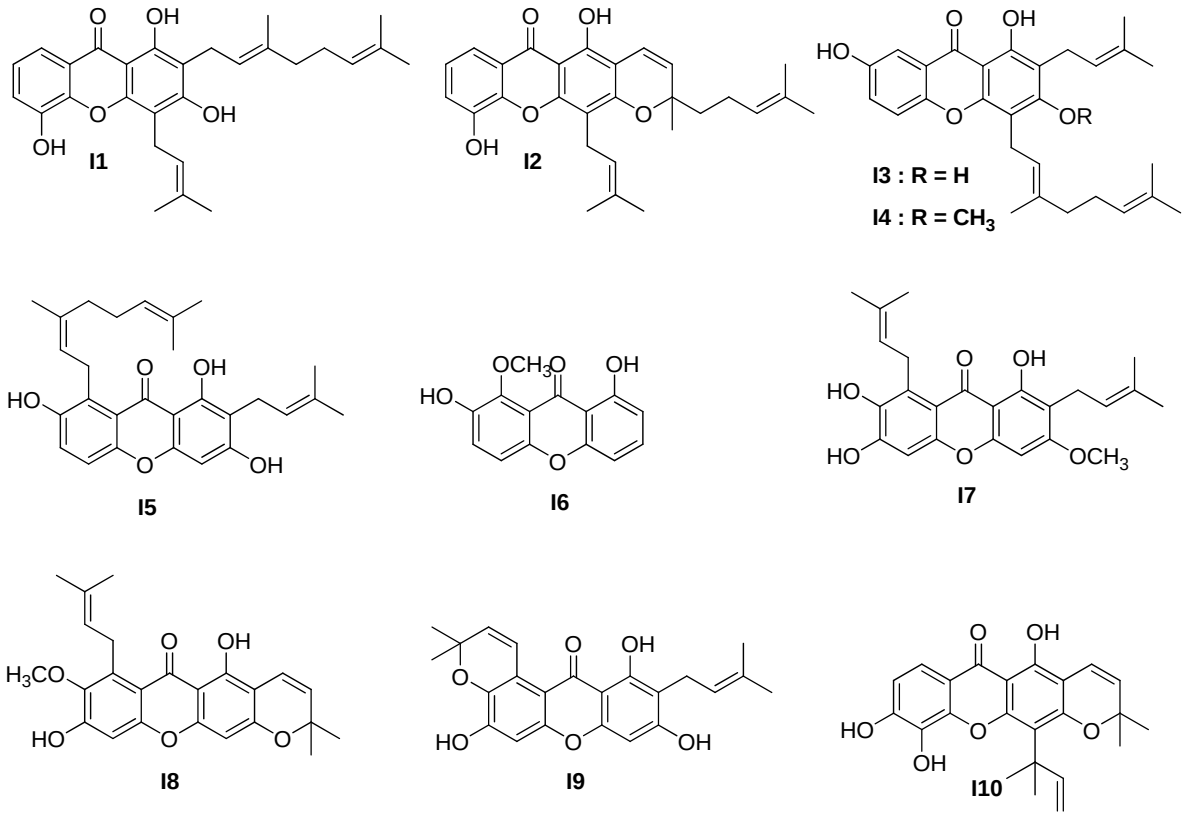


Scheme 3. Synthesis of β-mangostin analogs **6a**-**6f**

From the anti-bacterial activity result as shown in **Table 2**, the result showed that modified compounds **6b**-**6f** were inactive for anti-bacterial activity. From the unexpected results, no modified compounds exhibit potent anti-bacterial activity than their precursors but only modified compound **5a** showed strong activity against MRSA and *B. substilis* with MIC value of 2.34 μg/mL comparative to those α-mangostin. We decide to search for another useful activity of an isolated xanthones from *Cratoxylum formosum* ssp. *pruniflorum*.

The north-eastern people in Thailand have been used the young fresh leaves of this plant for cooking ingredient as well as vegetable side disk. Moreover, the *C. formosum* ssp. *pruniflorum* has been used as a folk medicine for the treatment of inflammation as skin wound healing (Maisuthisakul *et al*., 2007). Most importantly, some of isolated compounds

from *Cratoxylum* plants have been found to possess potent anti-inflammation (Chen *et al*., 2008; Boonnak *et al*., 2010). For the next study is aimed to investigate the bioactive substances responsible for NO inhibition from the CH_2Cl_2 crude extract of *C. formosum* ssp. *pruniflorum* roots.



Scheme 4. Isolated structures of compounds **I1**-**I10**

Using Anti-NO assay-guided separation of crude CH_2Cl_2 extract of *C. formosum* ssp. *pruniflorum* roots led to the isolation of ten known xanthones including formoxanthone A (**I1**) (Boonsri et al., 2006), pruniflorone K (**I2**) (Boonnak et al., 2010a), cochinchinone A (**I3**) (Mahabusarakam et al., 2008), pruniflorone L (**I4**) (Boonnak et al., 2010a), cochinxanthone E (**I5**) (Laphookhieo et al., 2009), 1,7-dihydroxy-8-methoxyxanthone (**I6**) (Kijjoa et al., 1998), dulxisxanthone F (**I7**) (Deachathai et al., 2005), 5,9-dihydroxy-8-methoxy-2,2-dimethyl-7-(3-methylbut-2-enyl)-2*H*,6*H*-pyrano-[3,2*b*]-xanthone (**I8**) (Sen et al., 1980), garcinone B (**I9**) (Sen et al., 1982) and a mixture of garcinone B (**I9**) and macruraxanthone (**I10**) (Boonnak et al., 2010a) (**Scheme 4**), which were further evaluated for their inhibitory effects on the LPS-induced nitric oxide production using murine macrophage RAW264.7 cells.

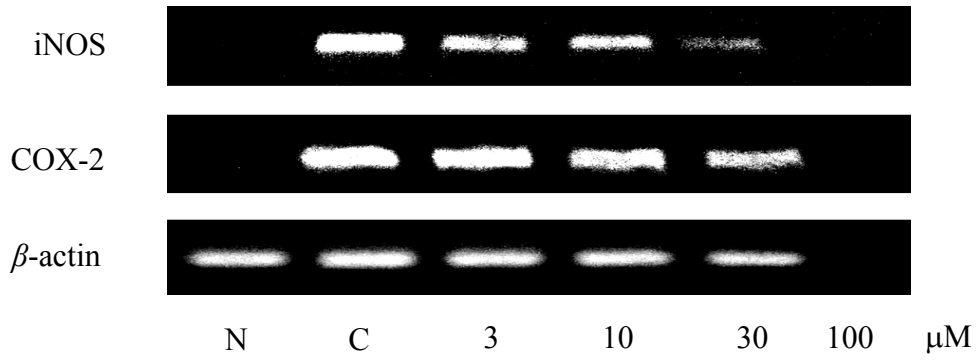
Table 3 Nitric oxide inhibitory activity of **I1-I9** and a mixture of **I9** and **I10**

Compounds	% inhibition of various concentrations (μM)[a]						IC_{50} (μM)
	0	1	3	10	30	100	
I1	0.0±3.3	–	22.1±1.6*	50.1±2.3**	97.9±0.6[b]**	101.0±3.6[b]**	8.0
I2	0.0±3.3	–	–	27.0±2.5*	62.1±3.2**	99.3±2.8**	20.6
I3	0.0±2.4	–	–	35.3±0.7**	90.4±1.2[b]**	105.1±3.7[b]**	12.6
I4	0.0±3.3	–	–	8.9±2.4	38.9±1.8**	94.9±3.9[b]**	33.1
I5	0.0±2.4	–	–	36.9±3.2**	86.3±1.0**	106.5±1.0[b]**	12.8
I6	0.0±3.3	–	–	17.7±2.5*	36.2±3.8**	90.8±0.7**	32.8
I7	0.0±3.3	12.6±2.5*	50.4±2.5**	78.8±0.4**	93.9±3.0**	101.7±1.2[b]**	3.9
I8	0.0±2.4	–	37.8±3.1**	67.1±1.9**	99.1±1.5**	100.9±1.7[b]**	4.3
I9	0.0±2.4	–	–	29.9±2.7**	109.0±1.4[b]**	109.6±2.1[b]**	11.8
I9+**I10**[c]	0.0±2.4	–	22.5±1.8*	104.5±2.0[b]**	106.2±1.8[b]**	106.0±1.4[b]**	12.4
Indomethacin	0.0±4.2	–	16.6±2.9	32.7±2.6**	53.4±3.0**	85.6±1.8**	20.1
L-NA	0.0±5.6	–	15.3±2.8	21.4±2.5	35.6±2.1**	73.2±3.5**	59.0
CAPE	0.0±5.6	–	35.2±3.0*	70.3±2.7**	97.6±2.4[b]**	99.5±2.7[b]**	5.0

[a] each value represents mean ± S.E.M. of four determinations.
[b] cytotoxic effect was observed.
[c] a 1:1 mixture of compounds **I9** and **I10**
statistical significance, * $p<0.05$, ** $p<0.01$

From the NO inhibitory activity result in **Table 3**, it revealed that compounds **I7** and **I8** exhibited the potent activity against NO release with an IC_{50} value of 3.9 and 4.3 μM, respectively, which are more potent activity than indomethacin (IC_{50} = 20.1 μM) using as the positive control. While compound **I1** also exhibited good activity against NO release with an IC_{50} value of 8.0 μM, whereas compounds **I3**, **I5** and **I9** showed moderate activity with an IC_{50} values of 12.6, 12.8 and 11.8 μM, respectively. In order to understand the mechanism of this anti-inflammatory activity, compounds **I7** and **I8** were further examined for their anti-inflammatory mechanisms against mRNA expressions of COX-2 and iNOS genes by using a semi-quantitative RT-PCR technique.

a) Compound **I7**



b) Compound **I8**

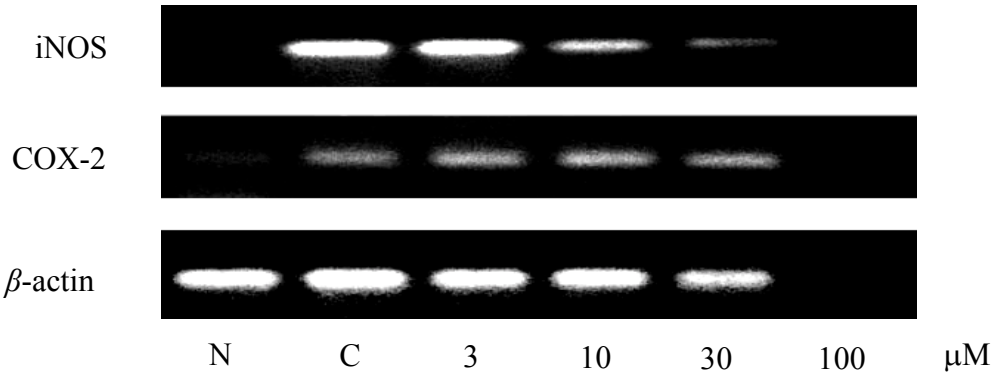


Figure 1. Effect of (**a**) compound **I7** and (**b**) compound **I8** at various concentrations (0, 3, 10, 30, 100 μM) on mRNA expressions of iNOS and COX-2 using RAW264.7 cells.

(N) = LPS (-), sample (-)

(C) = LPS (+), sample (-)

3-100 μM = LPS (+), sample (+)

The mechanisms in transcriptional level of **I7** was found to down regulate mRNA expressions of iNOS and COX-2 in dose-dependent manners, whereas **I8** inhibited only iNOS mRNA expression but did not affect on that of COX-2 gene (**Figure 2**). From this result, it could be suggested that compounds **I7** and **I8** are potent for anti-inflammatory activity by inhibition of NO production.

Conclusions

From the enhancing anti-bacterial activity and reducing their toxicity results, they revealed that no modified compounds exhibit potent anti-bacterial activity than their precursors but only modified compound **5a** showed strong activity against MRSA and *B. substilis* with MIC value of 2.34 μg/mL comparative to those α-mangostin. We decide to search for another useful activity of an isolated xanthones from *Cratoxylum formosum* ssp. *pruniflorum*.

The anti-inflammatory activity results showed that most of all isolated compounds showed anti-inflammatory effects ranging from 3.9 to 33.1 μM, especially for dulxisxanthone F (**I7**) and 5,9-dihydroxy-8-methoxy-2,2-dimethyl-7-(3-methylbut-2-enyl)-2*H*,6*H*-pyrano-[3,2*b*]-xanthone (**I8**). They showed potent anti-inflammatory activity with IC_{50} values of 3.9 and 4.3 μM, which higher than a positive controls; indomethacin (IC_{50} = 20.1 μM); L-NA (IC_{50} = 59.0 μM) and CAPE (IC_{50} = 5.0 μM). From the above mention, it could be suggested that *tetra*-oxygenated xanthone skeleton exhibit inhibition of NO production greater than *tri*-oxygenated xanthone skeleton, while a presence of a methoxyl group at C-3 (compound **I7**) or C-7 (compound **I8**) on the *tetra*-oxygenated isoprenylated-xanthone skeleton are essential for NO inhibitory activity compared to those compound **I9**. Compounds **I7** and **I8** are possible bioactive components responsible for anti-inflammatory activity in the *C. formosum* ssp. *pruniflorum*. This finding could be supported the traditional usage of this plant for the inflammation treatment.

Materials and Methods

General

Melting points were determined on the Fisher-John melting point apparatus. Optical rotations were measured on a JASCO P-1020 digital polarimeter. UV and IR spectra were recorded on SPECORD S 100 (Analytikjena) and Perkin-Elmer FTS FT-IR spectrophotometer, respectively. The ^{1}H and ^{13}C NMR spectra were recorded on a 300 MHz

Bruker FTNMR Ultra Shield^{TM} spectrometers in $CDCl_3$ with TMS as the internal standard. Chemical shifts are reported in δ (ppm) and coupling constants (J) are expressed in hertz. EI and HREI mass spectra were measured on a Kratos MS 25 RFA spectrometer. Quick column chromatography (QCC) and column chromatography (CC) were carried out on silica gel 60 F_{254} (Merck) and silica gel 100 (Merck), respectively.

Plant material

Hulks of *G. mangostana* were collected from Pattalung Province, Thailand in 2013. Roots of *C. formosum* ssp. *pruniflorum* were collected from Nong Khai Province, Thailand, in May, 2004. Identification was made by Prof. Puangpen Sirirugsa, Department of Biology, Faculty of Science, Prince of Songkla University and a specimen (No. 0012677) was deposited at Prince of Songkla University Herbarium.

Physical properties of all compounds

***Compound* 1**: *norathyriol.* Pale yellow powder, m.p. = 300 (decomposed). For ^1H (300 MHz, CD_3OD + $CDCl_3$) δ (ppm): 7.39 (1H, *br s*), 6.78 (1H, *br s*), 6.26 (1H, *d*, J = 2.3 Hz), 6.12 (1H, *d*, J = 2.3 Hz) (Noro et al., 1984; Don et al., 2004).

***Compound* 2**: *γ-mangostin.* Brown yellow solid. m.p. > 300 decomposed. For ^1H (300 MHz, CD_3OD + $CDCl_3$) δ (ppm) : 13.88 (1-OH, *br s*), 6.68 (1H, *br s*), 6.28 (1H, *br s*), 5.32 (1H, *br t*, J = 6.9 Hz), 5.28 (1H, *br t*, J = 6.9 Hz), 4.16 (2H, *d*, J = 6.9 Hz), 3.35 (2H, *d*, J = 6.9 Hz), 1.86 (3H, *s*), 1.80 (3H, *s*), 1.68 (6H, *s*) (Mahabusarakam & Wiriyachitra 1987).

***Compound* 3**: *Dulxisxanthone.* Yellow powder, m.p. 170-172 °C. For ^1H (300 MHz, $CDCl_3$) δ (ppm) : 13.44 (1-OH, *br s*), 6.81 (1H, *s*), 6.32 (1H, *s*), 5.31 (1H, *br t*, J = 6.9 Hz), 5.23 (1H, *br t*, J = 7.2 Hz), 4.33 (2H, *d*, J = 6.9 Hz), 3.90 (-OCH_3, *s*), 3.35 (2H, *d*, J = 7.2 Hz), 1.89 (3H, *s*), 1.80 (3H, *s*), 1.79 (3H, *s*), 1.68 (3H, *s*) (Dechathai *et al*., 2005).

***Compound* 5**: *α-mangostin.* Brown yellow powder, m.p. 180-182 °C. For ^1H (300 MHz, $CDCl_3$) δ (ppm) : 13.77 (1-OH, *br s*), 6.82 (1H, *s*), 6.29 (1H, *s*), 5.29 (1H, *m*), 5.26 (1H, *m*), 4.09 (2H, *d*, J = 6.0 Hz), 3.81 (-OCH_3, *s*), 3.45 (2H, *d*, J = 7.2 Hz), 1.84 (6H, *s*), 1.77 (3H, *s*), 1.69 (3H, *s*) (Mahabusarakam *et al*., 1987).

***Compound* 6**: *β-mangostin.* Pale yellow powder, m.p. 172-174 °C. For ^1H (300 MHz, $CDCl_3$) δ (ppm) : 13.41 (1-OH, *br s*), 6.82 (1H, *s*), 6.32 (1H, *s*), 5.26 (1H, *m*), 5.23 (1H, *m*),

4.09 (2H, *d*, *J* = 7.2 Hz), 3.90 (-OCH_3, *s*), 3.81 (-OCH_3, *s*) 3.35 (2H, *d*, *J* = 7.2 Hz), 1.83 (3H, *s*), 1.80 (3H, *s*), 1.68 (6H, *s*) (Mahabusarakam *et al*., 1987).

The methylated of γ-mangostin was prepared by treatment with MeI/K_2CO_3 in methanol to give compounds **4**, **7** and **8**, which were tested for their anti-bacterial activity.

***Compound* 4**: *6-O-methyl γ-mangostin.* Yellow viscous oil. For ^{1}H (300 MHz, $CDCl_3$) δ (ppm) : 13.86 (1-OH, *br s*), 6.74 (1H, *s*), 6.30 (1H, *s*), 5.71 (7-OH, *br s*), 5.32 (2H, *m*), 4.16 (2H, *d*, *J* = 6.9 Hz), 3.98 (-OCH_3, *s*), 3.43 (2H, *d*, *J* = 6.9 Hz), 1.82 (3H, *s*), 1.81 (3H, *s*), 1.77 (3H, *s*), 1.70 (3H, *s*) (Dharmaratne et al., 2013).

***Compound* 7**: *6-O-methyl α-mangostin.* Yellow powder. m.p. 94-96 For ^{1}H (300 MHz, $CDCl_3$) δ (ppm) : 13.82 (1-OH, *br s*), 6.72 (1H, *s*), 6.28 (1H, *s*), 5.30 (2H, *m*), 4.12 (2H, *d*, *J* = 6.9 Hz), 3.94 (-OCH_3, *s*), 3.80 (-OCH_3, *s*) 3.46 (2H, *d*, *J* = 6.9 Hz), 1.85 (6H, *s*), 1.78 (3H, *s*), 1.68 (6H, *s*) (Sudta et al., 2013).

***Compound* 8**: *3,6-Di-O-methyl α-mangostin.* Yellow powder. m.p. 93- 95 °C. For ^{1}H (300 MHz, $CDCl_3$) δ (ppm) : 13.40 (1-OH, *br s*), 6.63 (1H, *s*), 6.22 (1H, *s*), 5.17 (1H, *br t*, *J* = 6.6 Hz), 5.15 (1H, *br t*, *J* = 6.6 Hz), 4.04 (2H, *d*, *J* = 6.6 Hz), 3.87 (-OCH_3, *s*), 3.81 (-OCH_3, *s*), 3.71 (-OCH_3, *s*), 3.26 (2H, *d*, *J* = 6.6 Hz), 1.77 (3H, *s*), 1.72 (3H, *s*), 1.60 (6H, *s*) (Sudta et al., 2013).

***Compound* 5a**: *3-Isomangostin.* Yellow powder. m.p. 154-155 °C. For ^{1}H (300 MHz, $CDCl_3$) δ (ppm) : 13.70 (1-OH, s), 6.83 (1H, *s*), 6.24 (1H, *s*), 5.27 (1H, *br t*, *J* = 6.3 Hz), 4.10 (2H, *d*, *J* = 6.3 Hz), 3.80 (-OCH_3, *s*), 2.71 (2H, *t*, *J* = 6.9 Hz), 1.83 (2H, *t*, *J* = 6.9 Hz), 1.69 (3H, *s*), 1.59 (3H, *s*), 1.24 (6H, *s*) (Mahabusarakam & Wiriyachitra 1987; Boonnak et al., 2006).

***Compound* 5b**: 3,4-dihydro-5,9-dihydroxy-8-methoxy-7-(3-methoxy-3-methylbutyl)-2,2-dimethyl-2H,6H-pyrano-[3,2-b]xanthen-6-one. Yellow powder. m.p. 181-182 °C. For ^{1}H (300 MHz, $CDCl_3$) δ (ppm) : 13.59 (1-OH, s), 6.82 (1H, *s*), 6.22 (1H, *s*), 3.86 (-OCH_3, *s*), 3.42 (2H, *m*), 2.69 (2H, *t*, *J* = 6.6 Hz), 1.80 (4H, *m*), 1.37 (6H, *s*), 1.33 (6H, *s*) (Dutta et al., 1987; Boonnak et al., 2006).

***Compound* 5c**: 3,4-Dihydro-5,9-dihydroxy-8-methoxy-7-(3-methoxy-3-methyl-butyl)-2,2-dimethyl-2H,6H-pyrano[3,2b]xanthone. Yellow oil. For ^{1}H (300 MHz, $CDCl_3$) δ (ppm) : 13.90 (1-OH, *s*), 6.81 (1H, *s*), 6.23 (1H, *s*), 3.86 (-OCH_3, *s*), 3.39 (2H, *m*), 3.86 (-OCH_3, *s*), 2.71 (2H, *t*, *J* = 6.9 Hz), 1.84 (2H, *t*, *J* = 6.9 Hz), 1.75 (2H, *m*), 1.37 (6H, *s*), 1.30 (6H, *s*) (Dutta *et al*., 1987).

***Compound* 5d**: *1-Isomangostin hydrate.* Yellow powder. For ^{1}H (300 MHz, CD_3OD + $CDCl_3$) δ (ppm) : 6.61 (1H, *s*), 6.22 (1H, *s*), 3.83 (-OCH_3, *s*), 3.38 (2H, *m*), 2.66 (2H, *t*, *J* = 6.6 Hz), 1.82 (4H, *m*), 1.42 (6H, *s*), 1.34 (6H, *s*) (Ren *et al*., 2011).

***Compound* 5e**: *3,6-Di-O-3,3-dimethylallyl-α-mangostin.* Brown yellow oil. For ^{1}H (300 MHz, $CDCl_3$) δ (ppm) : 13.50 (1-OH, *br s*), 6.72 (1H, *s*), 6.30 (1H, *s*), 5.53 (1H, *m*), 5.50 (1H, *m*), 5.25 (2H, *m*), 4.64 (2H, *d*, *J* = 6.9 Hz), 4.58 (2H, *d*, *J* = 6.3 Hz), 4.12 (2H, *d*, *J* = 7.2 Hz), 3.79 (-OCH_3, *s*), 3.34 (2H, *d*, *J* = 7.2 Hz), 1.84 (3H, *s*), 1.81 (6H, *s*), 1.78 (6H, *s*), 1.76 (3H, *s*), 1.67 (6H, *s*) (Ha *et al*., 2009).

***Compound* 5f**: *3,6-Dibrosylate-α-mangostin.* Yellow oil. For ^{1}H (300 MHz, $CDCl_3$) δ (ppm) : 13.31 (1-OH, *br s*), 7.91 (2H, *d*, *J* = 8.7 Hz), 7.77 (2H, *d*, *J* = 8.4 Hz), 7.75 (2H, *d*, *J* = 8.4 Hz), 7.69 (2H, *d*, *J* = 8.7 Hz), 7.32 (1H, *s*), 6.86 (1H, *s*),5.01 (1H, *br t*, *J* = 6.0 Hz), 4.94 (1H, *br t*, *J* = 6.9 Hz), 4.00 (2H, *d*, *J* = 6.0 Hz), 3.66 (-OCH_3, *s*), 3.18 (2H, *d*, *J* = 6.9 Hz), 1.75 (3H, *s*), 1.70 (3H, *s*), 1.67 (3H, *s*), 1.64 (3H, *s*)

***Compound* 6a**: *3-O-Methyltetrahydro-α-mangostin.* Yellow powder. m.p. 179- 180 °C. For ^{1}H (300 MHz, $CDCl_3$) δ (ppm) : 13.61 (1-OH, *br s*), 6.80 (1H, *s*), 6.30 (1H, *s*), 3.88 (-OCH_3, *s*), 3.84 (-OCH_3, *s*), 3.33 (2H, *m*), 2.64 (2H, *m*), 1.76 (1H, m), 1.60 (1H, m), 1.48 (2H, m), 1.39 (2H, m), 1.00 (6H, *d*, *J* = 6.6 Hz), 0.96 (6H, *d*, *J* = 6.6 Hz) (Sudta et al., 2013).

***Compound* 6b**: *Pruniflorone C.* Yellow powder, m.p. 134-136 °C. For ^{1}H (300 MHz, CD_3OD + $CDCl_3$) δ (ppm) : 6.75 (1H, *s*), 6.31 (1H, *s*), 5.21 (1H, *br t*, *J* = 6.9 Hz), 3.90 (-OCH_3, *s*), 3.83 (-OCH_3, *s*), 3.37 (2H, *m*), 3.33 (2H, *d*, *J* = 6.9 Hz), 1.79 (3H, *s*), 1.77 (2H, *m*), 1.68 (3H, *s*), 1.32 (6H, *s*) (Boonnak *et al*., 2006).

***Compound* 6c**: *3-O-Methyl-1-Isomangostin.* Yellow powder, m.p. 281-283 °C. For ^{1}H (300 MHz, CD_3OD + $CDCl_3$) δ (ppm) : 6.62 (1H, *s*), 6.28 (1H, *s*), 5.27 (1H, *br t*, *J* = 6.6 Hz), 4.00 (2H, *d*, *J* = 6.6 Hz), 3.81 (-OCH_3, *s*), 3.69 (-OCH_3, *s*), 2.55 (2H, *t*, *J* = 6.9 Hz), 1.74 (2H, *t*, *J* = 6.9 Hz), 1.36 (3H, *s*), 1.28 (3H, *s*) (Yates & Bhat 1968; Boonnak *et al*., 2012).

***Compound* 6d**: *Pruniflorone A.* Yellow powder. m.p. 259-260 °C. For ^{1}H (300 MHz, $CDCl_3$) δ (ppm) : 6.79 (1H, *s*), 6.37 (1H, *s*), 3.90 (-OCH_3, *s*), 3.85 (-OCH_3, *s*), 3.41 (2H, *m*), 2.63 (2H, *t*, *J* = 6.6 Hz), 1.82 (4H, *m*), 1.40 (6H, *s*), 1.24 (6H, *s*) (Boonnak *et al*., 2006).

***Compound* 6e**: *Pruniflorone B.* Yellow powder. m.p. 215-217 °C. For ^{1}H (300 MHz, $CDCl_3$) δ (ppm) : 6.75 (1H, *s*), 6.42 (1H, *s*), 3.90 (-OCH_3, *s*), 3.83 (-OCH_3, *s*), 3.36 (2H, *m*), 3.33 (-OCH_3, *s*), 2.62 (2H, *t*, *J* = 6.6 Hz), 1.82 (4H, *m*), 1.41 (6H, *s*), 1.32 (6H, *s*) (Boonnak *et al*., 2006).

***Compound* 6f**: *Pruniflorone C.* Yellow powder. For ^{1}H (300 MHz, $CDCl_3$) δ (ppm) : 6.69 (1H, *s*), 6.27 (1H, *s*), 3.83 (-OCH_3, *s*), 3.74 (-OCH_3, *s*), 3.33 (2H, *m*), 2.56 (2H, *t*, *J* = 6.9 Hz), 2.32 (-OAc, *s*) 1.98, (2H, *t*, *J* = 6.9 Hz), 1.75 (2H, *m*), 1.35 (6H, *s*), 1.24 (6H, *s*) (Boonnak *et al*., 2006).

***Compound* I1**: *Formoxanthone A*. Yellow powder, mp 111-113 ^{o}C; UV-Vis ($CHCl_3$) λ_{max} (log ε) 245 (4.39), 269 (411), 332 (3.71), 377 (3.18) nm; FT-IR (KBr) ν_{max} 3373, 1650 cm^{-1}. ^{1}H MNR (300 MHz, $CDCl_3$): 13.20 (*s*, 1-OH), 6.57 (s, 3-OH), 5.86 (br s, 5-OH), 7.29 (dd, *J* = 7.8, 0.9 Hz, H-6), 7.22 (t, *J* = 7.8 Hz, H-7), 7.75 (dd, *J* = 7.8, 0.9 Hz, H-8), 3.50 (d, *J* = 7.2 Hz, H-1′), 5.29 (br t, *J* = 7.2 Hz, H-2′), 2.11 (m, CH_3-4′/5′), 5.06 (m, H_2-6′), 1.68 (s, CH_3-8′), 1.85 (s, CH_3-9′), 1.60 (s, CH_3-10′), 3.54 (d, *J* = 6.9 Hz, H_2-1′′), 5.26 (br t, *J* = 6.9 Hz, H-2′′), 1.74 (s, CH_3-4′′), 1.86 (s, CH_3-5′′) (Boonsri et al., 2006).

***Compound* I2**: *Pruniflorone K*. Yellow viscous oil, UV-Vis ($CHCl_3$) λ_{max} (log ε) 245 (4.63), 260 (4.56), 317 (4.37), 367 (3.73) nm; FT-IR (KBr) ν_{max} 3338, 1647, 1617 cm^{-1}; For ^{1}H NMR (300 MHz, $CDCl_3$): 13.08 (s, 1-OH), 5.73 (br s, 5-OH), 7.30 (dd, *J* = 7.8, 1.8 Hz, H-6), 7.23 (t, *J* = 7.8 Hz, H-7), 7.75 (dd, *J* = 7.5, 1.8 Hz, H-8), 6.79 (d, *J* = 10.2 Hz, H-1′), 5.56 (d, *J* = 10.2 Hz, H-2′), 1.78 (m, H_2-4′), 2.12 (m, H_2-5′), 5.09 (brt, *J* = 6.9 Hz, H-6′), 1.68 (s, CH_3-8′), 1.45 (s, CH_3-9′), 1.45 (s, CH_3-10′), 3.50 (d, *J* = 6.9 Hz, H2-1′′), 5.23 (br t, *J* = 6.9 Hz, H-2′′), 1.72 (s, CH_3-4′′), 1.84 (s, CH_3-5′′) (Boonnak et al., 2010a).

***Compound* I3**: *Cochinchinone A*. Pale-yellow powder, m.p. 119-120 °C; UV-Vis ($CHCl_3$) λ_{max} (log ε) 232 (4.44), 268 (4.42), 316 (4.04), 384 (3.70) nm; FT-IR (neat) ν_{max} 3413, 1641 cm^{-1}; For ^{1}H NMR (300 MHz, $CDCl_3$): 12.79 (s, 1-OH), 7.04 (d, *J* = 9.0 Hz, H-5), 7.07 (brd, *J* = 9.0 Hz, H-6), 7.44 (br s, H-8), 3.32 (d, *J* = 6.9 Hz, H_2-1′), 5.20 (br t, *J* = 6.9 Hz, H-2′), 1.55 (s, CH_3-4′), 1.75 (s, CH_3-5′), 3.39 (d, *J* = 6.9 Hz, H_2-1′′), 5.16 (br t, *J* = 6.9 Hz, H-2′′), 1.99 (m, H_2-4′′), 1.97 (m, H_2-5′′), 4.96 (br t, *J* = 7.2 Hz, H-6′′), 1.67 (s, CH_3-8′′), 1.78 (s, CH_3-9), 1.48 (s, CH_3-10′′) (Mahabusarakam et al., 2008).

***Compound* I4**: *Pruniflorone L.* Pale yellow powder, mp 259-260 ^{o}C; UV-Vis ($CHCl_3$) λ_{max} (log ε) 245 (4.05), 268 (4.19), 317 (3.81), 388 (3.39) nm; FT-IR (KBr) ν_{max} 3421, 1637 cm^{-1}; For ^{1}H NMR (300 MHz, $CDCl_3$): 12.78 (s, 1-OH), 7.28 (d, *J* = 8.7 Hz, H-5), 7.50 (dd, *J* = 8.7, 1.8 Hz, H-6), 6.55 (br s, 7-OH), 7.54 (d, *J* = 1.8 Hz, H-8), 3.34 (d, *J* = 6.6 Hz, H_2-1′), 5.20 (br t, *J* = 6.6 Hz, H-2′), 1.63 (s, CH_3-4′′), 1.74 (s, CH_3-5′′), 3.36 (d, *J* = 6.9 Hz, H_2-1′′), 5.15 (br t, *J* = 6.9 Hz, H-2′′), 1.94 (m, H_2-4′′), 1.98 (m, H_2-5′′), 4.95 (br t, *J* = 6.6 Hz, H-6′′),

1.50 (s, CH_3-8″), 1.80 (s, CH_3-9″), 1.46 (s, CH_3-10″), 3.74 (s, 3-OCH_3) (Boonnak et al., 2010a).

***Compound* I5**: *Cochinxanthone E.* Yellow oil; UV-Vis (CH_3OH) λ_{max} (log ε) 241 (4.20), 265 (4.18), 314 (3.92), 382 (3.43) nm; FT-IR (neat) ν_{max} 3437, 1638 cm^{-1}. For ^1H NMR (300 MHz, $CDCl_3$): 13.44 (s, 1-OH), 6.24 (s, H-4), 7.13 (s, H-5/6), 3.45 (d, *J* = 7.2 Hz, H_2-1′), 5.23 (br t, *J* = 7.2 Hz, H-2′), 1.70 (s, CH_3-4′), 1.78 (s, CH_3-5′), 4.25 (d, *J* = 6.9 Hz, H_2-1″), 5.20 (br t, *J* = 7.2 Hz, H-2″), 2.02 (m, H_2-4″/5″), 4.97 (br t, J = 6.0 Hz, H-6″), 1.59 (s, CH_3-8″), 1.80 (s, CH_3-9″), 1.51 (s, CH_3-10″) (Laphookhieo et al., 2009).

***Compound* I6**: *1,7-dihydroxy-8-methoxyxanthone*. Yellow solid, mp 197-199 ^oC; UV-Vis (NaOH) λ_{max} 254, 275, 350 nm; FT-IR (KBr) ν_{max} 3330, 1647 cm^{-1}. For ^1H NMR (300 MHz, $CDCl_3$): 12.82 (s, 1-OH), 6.70 (d, *J* = 8.4 Hz, H-2), 7.49 (t, *J* = 8.4 Hz, H-3), 6.80 (br d, *J* = 8.4 Hz, H-4), 7.14 (d, *J* = 9.3 Hz, H-5), 7.36 (dd, *J* = 9.3 Hz, H-6), 3.97 (s, 8-OCH_3) (Kijjoa et al., 1998).

***Compound* I7**: *Dulcisxanthone B.* Yellow powder, mp 170-172 ^oC; UV-Vis (CH_3OH) λ_{max} (log ε) 209 (4.25), 244 (4.50), 261 (4.49), 317 (4.25) 368 (4.04) nm; FT-IR (KBr) ν_{max} 3306, 1642 cm^{-1}. For ^1H NMR (300 MHz, $CDCl_3$): 13.44 (s, 1-OH), 6.32 (s, H-4), 6.81 (s, H-5), 3.35 (d, *J* = 7.2 Hz, H_2-1′), 5.23 (br t, *J* = 7.2 Hz, H-2′), 1.68 (s, CH_3-4′), 1.80 (s, CH_3-5′), 4.33 (d, *J* = 6.9 Hz, H_2-1″), 5.31 (br t, *J* = 6.9 Hz, H-2″), 1.79 (s, CH_3-4″), 1.89 (s, CH_3-5″), 3.90 (s, 3-OCH_3) (Deachathai et al., 2005).

***Compound* I8**: *5,9-dihydroxy-8-methoxy-2,2-dimethyl-7-(3-methyl-but-2-enyl)-2H,-6H-pyrano-[3,2b]-xanthone.* Yellow powder, mp 156-157 ^oC. For ^1H NMR (300 MHz, $CDCl_3$):13.63 (s, 1-OH), 6.17 (s, H-4), 6.81 (s, H-5), 6.66 (d, *J* = 9.9 Hz, H-1′), 5.50 (d, *J* = 9.9 Hz, H-2′), 1.39 (s, CH_3-4′), 1.39 (s, CH_3-5′), 4.01 (d, *J* = 6.3 Hz, H_2-1″), 5.19 (br t, *J* = 6.6 Hz, H-2″), 1.62 (s, CH_3-4″), 1.76 (s, CH_3-5″), 3.73 (s, 7-OCH_3) (Sen et al., 1980).

***Compound* I9**: *Garcinone B.* Yellow powder, mp 190-192 ^oC; UV-Vis (EtOH) λ_{max} (log ε) 247 (4.40), 267 (4.40), 339 (4.10), 390 (4.00) nm; FT-IR (KBr) ν_{max} 3480, 1650 cm^{-1}. For ^1H NMR (300 MHz, $CDCl_3$): 13.62 (s, 1-OH), 6.25 (s, H-4), 6.74 (s, H-5), 3.38 (d, *J* = 6.6 Hz, H_2-1′), 5.23 (br t, *J* = 6.6 Hz, H-2″), 1.70 (s, CH_3-4′), 1.77 (s, CH_3-5′), 7.95 (d, *J* = 10.2 Hz, H-1″), 5.75 (d, *J* = 10.2 Hz, H-2″), 1.43 (s, CH_3-4″/5″) (Sen et al., 1982).

A mixture of ***Compound* I9** and ***Compound* I10** (macluraxanthone). For ^1H NMR (300 MHz, $CDCl_3$): 13.62 (s, 1-OH), 6.25 (s, H-4), 6.74 (s, H-5), 3.38 (d, *J* = 6.6 Hz, H_2-1′), 5.23 (br t, *J* = 6.6 Hz, H-2″), 1.70 (s, CH_3-4′), 1.77 (s, CH_3-5′), 7.95 (d, *J* = 10.2 Hz, H-1″),

5.75 (d, *J* = 10.2 Hz, H-2″), 1.43 (s, CH_3-4″/5″) ;13.53 (s, 1-OH), 6.94 (d, *J* = 9.0 Hz, H-7), 7.68 (d, *J* = 9.0 Hz, H-8), 6.76 (d, *J* = 9.9 Hz, H-1′), 5.61 (d, *J* = 9.9 Hz, H-2′), 1.52 (s, CH_3-4′/5′), 6.76 (dd, *J* = 17.7, 10.5 Hz, H-2″), 5.22 (dd, *J* = 17.7, 1.5 Hz, H-3″), 5.05 (dd, *J* = 10.5, 1.5 Hz, H-3″), 1.65 (s, CH_3-4″/5″) (Boonnak et al., 2010a; Sen et al., 1982).

Anti-bacterial Assay

Compounds **1-8** and modified compounds **5a-5f** and **6a-6f** were tested against both Gram-positive and Gram-negative bacteria: *B. subtilis*, *S. aureus*, TISTR517, *E. faecalis* TISTR459, Methicillin-Resistant *S. aureus* (MRSA) ATCC43300, Vancomycin-Resistant *E. faecalis* (VRE) ATCC 51299, *Streptococcus faecalis*, *S. typhi*, *S. sonei* and *P. aeruginosa*. The microrganisms were obtained from the culture collections, Department of Industrial Biotechnology and Department of Pharmacognosy and Botany, PSU, except for the TISTR and ATCC strains, which were obtained from Microbial Research Center (MIRCEN), Bangkok, Thailand. The antimicrobial assay employed was the colorimetric microdilution broth technique using RPMI1640 medium and Alamar Blue as an indicator (Kanjana-Opas, A., 2002; Boonsri *et al*., 2006). Microbial inocula were prepared as suspension in RPMI1640 medium and mixed with 1% 100× Alamar Blue indicator. The cell suspension was then transferred into a 96-well microliter plate (100 μL/well except for first row which contained 190 μL/ well). Sample compounds (10 μL) dissolved in DMSO at a concentration of 25 mg/mL was added to each well of the first row and mixed well with a micropipette. Half of the mixtures of cell suspension and extracts in the first rows were then transferred to the next well in the second row to perform a half-fold dilution. The dilution process was repeated as a sequence until the extracts were diluted 128 times in the last row. The excess 100 μL of the mixture in the last row was discarded. The plates were incubated at 37 °C for 8–12 h and 24 h for antibacterial and antifungal assays, respectively. The antimicrobial activity was determined as the MIC value which was the least concentration of the extract that could inhibit the change of Alamar Blue indicator from blue to red. Vancomycin, which was used as a standard, showed antibacterial activity against Vancomycin-Resistant E. faecalis (VRE) ATCC 51299 at 75.0 mg/mL.

NO production from RAW264.7 macrophages stimulated by LPS

Inhibitory effect on NO production by murine macrophage-like RAW264.7 cells was evaluated using a modified method from that previously reported (Banskota et al., 2003). Briefly, the RAW264.7 cell line (purchased from Cell Lines Services) was cultured in RPMI medium supplemented with 0.1% sodium bicarbonate and 2 mM glutamine, penicillin G (100 units/mL), streptomycin (100 μg/mL) and 10% FCS. The cells were harvested with trypsin-EDTA and diluted to a suspension in a fresh medium. The cells were seeded in 96-well plates with 1×10^5 cells/well and allowed to adhere for 1 h at 37°C in a humidified atmosphere containing 5% CO_2. After that the medium was replaced with a fresh medium containing 50 μg/mL of LPS together with the test samples at various concentrations (3-100 μg/mL for crude extract and 3-100 μM for pure compounds) and was then incubated for 24 h. NO production was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent. Cytotoxicity was determined using the MTT colorimetric method. Briefly, after 24 h incubation with the test samples, MTT solution (10 μL, 5 mg/mL in PBS) was added to the wells. After 4 h incubation, the medium was removed, and isopropanol containing 0.04 M HCl was then added to dissolve the formazan production in the cells. The optical density of the formazan solution was measured with a microplate reader at 570 nm. The test compounds were considered to be cytotoxic when the optical density of the sample-treated group was less than 80% of that in the control (vehicle-treated) group. L-NA, CAPE and indomethacin were used as positive controls. The stock solution of each test sample was dissolved in DMSO, and the solution was added to the medium RPMI (final DMSO is 1%). Inhibition (%) was calculated using the following equation and IC_{50} values were determined graphically (n = 4):

$$\text{Inhibition (\%)} = \frac{A - B}{A - C} \times 100$$

A-*C* : NO_2^- concentration (μM) [*A* : LPS (+), sample (-); *B* : LPS (+), sample(+); *C* : LPS (-), sample (-)].

Total RNA isolation and RT-PCR

In order to acquire the mechanism of action on cytokine release of compounds **7** and **8** the assays for mRNA expression of iNOS and COX-2 were carried out. The total RNA was isolated from RAW264.7 cells and was harvested after 20 h of incubation with samples in various concentrations (3, 10, 30, 100 μM) using the RNeasy Mini Kit (Qiagen Operon Co. Ltd., USA). The total RNA from each sample was used for cDNA synthesis using first strand cDNA synthesis kit (Rever Tra Ace-α, TOYOBO Co., Ltd., Japan), followed by RT-PCR (Rever Tra Dash, TOYOBO Co., Ltd., Japan). The primers for iNOS and COX-2 were used (forward primer for iNOS : 5′-ATCTGGATCAGGAACCTGAA-3′ and its reverse primer: 5′-CCTTTTTTGCCCCATAGGAA-3′; forward primer for COX-2: 5′-GGAGAGACTATCAAGATAGTGATC-3′ and its reverse primer: 5′-ATGGTCAGTAGACTTTTACAGCTC-3′; forward primer for β-actin (an internal standard): 5′-TGTGATGGTGGGAATGGGTCAG-3′ and reverse primer: 5′-TTTGATGTCACGCACGATTTCC-3′.

The solution for cDNA synthesis consisted of RNA solution 11 μL, 5 x RT buffer 4 μL, dNTP mixture (10 mM) 2 μL, RNase inhibitor (10 U/μL) 1 μL, Oligo(dT)20 1 μL and Rever Tra Ace (reverese transcriptase enzyme) 1 μL for a 20 μL reaction. The condition for cDNA synthesis was as follow; 42 °C for 20 min, 99 °C for 5 min and 4 °C for 5 min. After that, 1/10 times (2 μL) of cDNA product was used further for PCR. The PCR mixture consisted of RT reaction mixture (cDNA product) 2 μL; sterilized water 85 μL, 10 x PCR buffer 10 μL, forward primer (10 pmol/μL) 1 μL, reverse primer (10 pmol/μL) 1 μL and KOD Dash (polymerase enzyme)1 μL for final volume of 100 μL. The condition for PCR was as follow; denaturation at 94 °C for 1 min, 98 °C for 30 s, 55 °C for 30 s and 74 °C for 1 min (30 cycles). The PCR products were analyzed in 1.2 % agarose gel electrophoresis and visualized by SYBR safe staining and UV irradiation under a wavelength of 312 nm.

Statistics

For statistical analysis, the values are expressed as mean ± S.E.M of four determinations. The IC_{50} values were calculated using the microsoft excel programme. The statistical significance was calculated by one-way analysis of variance (ANOVA), followed by Dunnett's test.

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Appendix

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RESEARCH ARTICLE

Inhibition of nitric oxide production in lipopolysaccharide-activated RAW264.7 macrophages by isolated xanthones from the roots of *Cratoxylum formosum* ssp. *pruniflorum*

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Abstract The inhibitory activity of extract and compounds isolated from the roots of *Cratoxylum formosum* ssp. *pruniflorum* against nitric oxide (NO) was evaluated using RAW264.7 cells. Isolation of the CH_2Cl_2 extract of *C. formosum* ssp. *pruniflorum* roots afforded ten known xanthones including six *tri*-oxygenated xanthones (**1**–**6**) and four *tetra*-oxygenated xanthones (**7**–**10**), respectively. Compound **7** showed the highest inhibitory activity against NO release with an IC_{50} value of 3.9 μM, followed by compound **8** with an IC_{50} value of 4.3 μM, respectively. In order to understand the mechanism of this anti-inflammatory activity, the transcriptional level of **7** was found to down regulate mRNA expressions of iNOS and COX-2 in dose-dependent manners, whereas **8** inhibited only iNOS mRNA expression but did not affect on that of COX-2 gene. Xanthones might be the main anti-inflammatory components in *C. formosum* ssp. *pruniflorum*.

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Keywords Nitric oxide inhibition · Xanthones · *Cratoxylum formosum* ssp. *pruniflorum* cyclooxygenase-2 (COX-2) · Inducible nitric oxide synthase (iNOS)

Introduction

Cratoxylum formosum ssp. *pruniflorum* is a shrub tree producing small bright yellow flower belonging to the Guttiferae family (Smitinand 2001) and widely distributed around the Southeast Asia particularly around the northern zone of Southeast Asian such as Laos, Cambodia and Thailand. This plant has been used as a folk medicine for the treatment of inflammation as skin wound healing (Maisuthisakul et al. 2007). Many phytochemical studies on *Cratoxylum* species have revealed a series of anthraquinone (Boonnak et al. 2006), xanthones (Bennett et al. 1993; Ilnuma et al. 1996; Mahabusarakam et al. 2006; Boonnak et al. 2009; Duan et al. 2011), flavonoids (Kitanov et al. 1988) and triterpenoids (Reutrakul et al. 2006). Some of isolated compounds have been found to possess potent bioactivity (Boonnak et al. 2006, 2007; Reutrakul et al. 2006; Mahabusarakam et al. 2008) such as anti-inflammatory activity (Chen et al. 2008; Boonnak et al. 2010b). It is well documented that inflammation is becoming serious problems of human being. The key factor of inflammation is a rapid massive releasing of nitric oxide (NO) (Mordan et al. 1993; Ohshima and Bartsch 1994; Kronche et al. 1998; Tewtrakul and Subhadhirasakul 2008; Sudsai et al. 2013). This prompted us to search for bioactive compounds from Thai herb with responsible for anti-inflammation. In our present research, it investigated the bioactive substances responsible for NO inhibition from the CH_2Cl_2 crude extract of *C. formosum* ssp. *pruniflorum* roots.

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Materials and methods

General

Melting points were determined on the Fisher-John melting point apparatus. Optical rotations were measured on a JASCO P-1020 digital polarimeter. UV and IR spectra were recorded on SPECORD S 100 (Analytikjena) and Perkin-Elmer FTS FT-IR spectrophotometer, respectively. The ^{1}H and ^{13}C NMR spectra were recorded on a 300 MHz Bruker FTNMR Ultra Shield^{TM} spectrometers in $CDCl_3$ with TMS as the internal standard. Chemical shifts are reported in δ (ppm) and coupling constants (J) are expressed in hertz. EI and HREI mass spectra were measured on a Kratos MS 25 RFA spectrometer. Quick column chromatography (QCC) and column chromatography (CC) were carried out on silica gel 60 F_{254} (Merck) and silica gel 100 (Merck), respectively.

Plant material

Roots of *C. formosum* ssp. *pruniflorum* were collected from Nong Khai Province, Thailand, in May, 2004. Identification was made by Prof. Puangpen Sirirugsa, Department of Biology, Faculty of Science, Prince of Songkla University and a specimen (No. 0012677) was deposited at Prince of Songkla University Herbarium.

Extraction and isolation

The CH_2Cl_2 crude extract (58.87 g) of the roots of *C. formosum* spp. *pruniflorum* was subjected to QCC on silica gel using *n*-hexane as a first eluent and then increasing the polarity with acetone to give 12 fractions (FR1–FR12). Fractions FR6 and FR7 were separated by QCC eluting with a gradient of acetone–*n*-hexane to give 10 subfractions (FR6A–FR6J). Subfraction FR6B was separated by QCC and eluted with a gradient of acetone-*n*-hexane to give 11 subfractions (FR6B1–FR6B11). Subfractions FR6B2 were separated by CC and eluted with 10 % acetone–*n*-hexane to give 4 subfractions (FR6B2A–FR6B2D) and formoxanthone A (**1**) (25.5 mg). Subfractions FR6B6 and FR6B7 were separated by CC and eluted with 30 % CH_2Cl_2–*n*-hexane to give 9 subfractions (FR6B6A–FR6B6I) and pruniflorone K (**2**) (5.7 mg). Subfraction FR6B6E was further purified by CC on silica gel C-18 and eluted with MeOH to furnish 5,9-dihydroxy-8-methoxy-2,2-dimethyl-7-(3-methylbut-2-enyl)-2*H*,6*H*-pyrano-[3,2*b*]-xanthone (**8**) (7.0 mg). Subfraction FR6H was further separated by QCC eluting with a gradient of acetone–*n*-hexane to give 11 subfractions (FR6H1–FR6H11), cochinchinone A (**3**) (80.7 mg) and pruniflorone L (**4**) (9.7 mg). Fractions FR8–FR11 was separated by QCC eluting with 30 % EtOAc–*n*-hexane to give 8 subfractions (FR8A–FR8H). Subfractions FR8E and FR8F were separated by QCC and eluted with 30 % EtOAc–*n*-hexane to obtain 20 subfractions (FR8E1–FR8E20). Subfraction FR8E8 was separated by CC eluting with acetone–*n*-hexane to give 1,7-dihydroxy-8-methoxyxanthone (**6**) (7.5 mg). Subfraction FR8E9–FR8E11 were separated by CC and eluted with a gradient of acetone–*n*-hexane to give dulxisxanthone F (**7**) (15.6 mg). Subfraction FR8E10–FR8E12 were separated by QCC and eluted with a gradient of CH_2Cl_2–*n*-hexane to give 12 subfractions (FR8E10A–FR8E10L). Subfraction FR8E10E was separated by CC and eluted with a gradient of acetone-*n*-hexane to give 7 subfractions (FR8E10E1–FR8E10E7) and cochinxanthone E (**5**) (2.5 mg). Subfraction FR8E10F was separated by CC and eluted with a gradient of acetone-*n*-hexane to give 8 subfractions (FR8E10F1–FR8E10F8). Subfraction FR8E10F6 was further separated by CC and eluted with 60 % $CHCl_3$-*n*-hexane to give 4 subfractions (FR8E10F6A–FR8E10F6D) and a mixture of **9** and **10** (35.5 mg), which was further purified by CC on reversed-phase silica gel C-18 eluting with MeOH to give garcinone B (**9**) (12.0 mg) and a mixture of garcinone B and macruraxanthone (**10**) (21.2 mg). The structures of the isolated compounds were elucidated and compared with the previous literatures.

*Formoxanthone A (**1**)*

Yellow powder, mp 111–113 °C; UV–Vis ($CHCl_3$) λ_{max} (log ε) 245 (4.39), 269 (411), 332 (3.71), 377 (3.18) nm; FT-IR (KBr) ν_{max} 3373, 1650 cm^{-1}. ^{1}H MNR (300 MHz, $CDCl_3$): 13.20 (s, 1-OH), 6.57 (s, 3-OH), 5.86 (br s, 5-OH), 7.29 (dd, $J = 7.8$, 0.9 Hz, H-6), 7.22 (t, $J = 7.8$ Hz, H-7), 7.75 (dd, $J = 7.8$, 0.9 Hz, H-8), 3.50 (d, $J = 7.2$ Hz, H-1′), 5.29 (br t, $J = 7.2$ Hz, H-2′), 2.11 (m, CH_3-4′/5′), 5.06 (m, H_2-6′), 1.68 (s, CH_3-8′), 1.85 (s, CH_3-9′), 1.60 (s, CH_3-10′), 3.54 (d, $J = 6.9$ Hz, H_2-1″), 5.26 (br t, $J = 6.9$ Hz, H-2″), 1.74 (s, CH_3-4″), 1.86 (s, CH_3-5″).

*Pruniflorone K (**2**)*

Yellow viscous oil, UV–Vis ($CHCl_3$) λ_{max} (log ε) 245 (4.63), 260 (4.56), 317 (4.37), 367 (3.73) nm; FT-IR (KBr) ν_{max} 3338, 1647, 1617 cm^{-1}; For ^{1}H NMR (300 MHz, $CDCl_3$): 13.08 (s, 1-OH), 5.73 (br s, 5-OH), 7.30 (dd, $J = 7.8$, 1.8 Hz, H-6), 7.23 (t, $J = 7.8$ Hz, H-7), 7.75 (dd, $J = 7.5$, 1.8 Hz, H-8), 6.79 (d, $J = 10.2$ Hz, H-1′), 5.56 (d, $J = 10.2$ Hz, H-2′), 1.78 (m, H_2-4′), 2.12 (m, H_2-5′), 5.09 (brt, $J = 6.9$ Hz, H-6′), 1.68 (s, CH_3-8′), 1.45 (s, CH_3-9′), 1.45 (s, CH_3-10′), 3.50 (d, $J = 6.9$ Hz, H2-1″), 5.23 (br t, $J = 6.9$ Hz, H-2″), 1.72 (s, CH_3-4″), 1.84 (s, CH_3-5″).

*Cochinchinone A (**3**)*

Pale-yellow powder, m.p. 119–120 °C; UV–Vis ($CHCl_3$) λ_{max} (log ε) 232 (4.44), 268 (4.42), 316 (4.04), 384 (3.70) nm; FT-IR (neat) ν_{max} 3413, 1641 cm^{-1}; For ^1H NMR (300 MHz, $CDCl_3$): 12.79 (s, 1-OH), 7.04 (d, $J = 9.0$ Hz, H-5), 7.07 (br d, $J = 9.0$ Hz, H-6), 7.44 (br s, H-8), 3.32 (d, $J = 6.9$ Hz, H_2-1′), 5.20 (br t, $J = 6.9$ Hz, H-2′), 1.55 (s, CH_3-4′), 1.75 (s, CH_3-5′), 3.39 (d, $J = 6.9$ Hz, H_2-1″), 5.16 (br t, $J = 6.9$ Hz, H-2″), 1.99 (m, H_2-4″), 1.97 (m, H_2-5″), 4.96 (br t, $J = 7.2$ Hz, H-6″), 1.67 (s, CH_3-8″), 1.78 (s, CH_3-9), 1.48 (s, CH_3-10″).

*Pruniflorone L (**4**)*

Pale-yellow powder, mp 259–260 °C; UV–Vis ($CHCl_3$) λ_{max} (log ε) 245 (4.05), 268 (4.19), 317 (3.81), 388 (3.39) nm; FT-IR (KBr) ν_{max} 3421, 1637 cm^{-1}; For ^1H NMR (300 MHz, $CDCl_3$): 12.78 (s, 1-OH), 7.28 (d, $J = 8.7$ Hz, H-5), 7.50 (dd, $J = 8.7$, 1.8 Hz, H-6), 6.55 (br s, 7-OH), 7.54 (d, $J = 1.8$ Hz, H-8), 3.34 (d, $J = 6.6$ Hz, H_2-1′), 5.20 (br t, $J = 6.6$ Hz, H-2′), 1.63 (s, CH_3-4″), 1.74 (s, CH_3-5″), 3.36 (d, $J = 6.9$ Hz, H_2-1″), 5.15 (br t, $J = 6.9$ Hz, H-2″), 1.94 (m, H_2-4″), 1.98 (m, H_2-5″), 4.95 (br t, $J = 6.6$ Hz, H-6″), 1.50 (s, CH_3-8″), 1.80 (s, CH_3-9″), 1.46 (s, CH_3-10″), 3.74 (s, 3-OCH_3).

*Cochinxanthone E (**5**)*

Yellow oil; UV–Vis (CH_3OH) λ_{max} (log ε) 241 (4.20), 265 (4.18), 314 (3.92), 382 (3.43) nm; FT-IR (neat) ν_{max} 3437, 1638 cm^{-1}. For ^1H NMR (300 MHz, $CDCl_3$): 13.44 (s, 1-OH), 6.24 (s, H-4), 7.13 (s, H-5/6), 3.45 (d, $J = 7.2$ Hz, H_2-1′), 5.23 (br t, $J = 7.2$ Hz, H-2′), 1.70 (s, CH_3-4′), 1.78 (s, CH_3-5′), 4.25 (d, $J = 6.9$ Hz, H_2-1″), 5.20 (br t, $J = 7.2$ Hz, H-2″), 2.02 (m, H_2-4″/5″), 4.97 (br t, $J = 6.0$ Hz, H-6″), 1.59 (s, CH_3-8″), 1.80 (s, CH_3-9″), 1.51 (s, CH_3-10″).

*1,7-Dihydroxy-8-methoxyxanthone (**6**)*

Yellow solid, mp 197–199 °C; UV–Vis (NaOH) λ_{max} 254, 275, 350 nm; FT-IR (KBr) ν_{max} 3330, 1647 cm^{-1}. For ^1H NMR (300 MHz, $CDCl_3$): 12.82 (s, 1-OH), 6.70 (d, $J = 8.4$ Hz, H-2), 7.49 (t, $J = 8.4$ Hz, H-3), 6.80 (br d, $J = 8.4$ Hz, H-4), 7.14 (d, $J = 9.3$ Hz, H-5), 7.36 (dd, $J = 9.3$ Hz, H-6), 3.97 (s, 8-OCH_3).

*Dulcisxanthone B (**7**)*

Yellow powder, mp 170–172 °C; UV–Vis (CH_3OH) λ_{max} (log ε) 209 (4.25), 244 (4.50), 261 (4.49), 317 (4.25) 368 (4.04) nm; FT-IR (KBr) ν_{max} 3306, 1642 cm^{-1}. For ^1H NMR (300 MHz, $CDCl_3$): 13.44 (s, 1-OH), 6.32 (s, H-4), 6.81 (s, H-5), 3.35 (d, $J = 7.2$ Hz, H_2-1′), 5.23 (br t, $J = 7.2$ Hz, H-2′), 1.68 (s, CH_3-4′), 1.80 (s, CH_3-5′), 4.33 (d, $J = 6.9$ Hz, H_2-1″), 5.31 (br t, $J = 6.9$ Hz, H-2″), 1.79 (s, CH_3-4″), 1.89 (s, CH_3-5″), 3.90 (s, 3-OCH_3).

*5,9-Dihydroxy-8-methoxy-2,2-dimethyl-7-(3-methyl-but-2-enyl)-2H,6H-pyrano-[3,2b]-xanthone (**8**)*

Yellow powder, mp 156–157 °C. For ^1H NMR (300 MHz, $CDCl_3$):13.63 (s, 1-OH), 6.17 (s, H-4), 6.81 (s, H-5), 6.66 (d, $J = 9.9$ Hz, H-1′), 5.50 (d, $J = 9.9$ Hz, H-2′), 1.39 (s, CH_3-4′), 1.39 (s, CH_3-5′), 4.01 (d, $J = 6.3$ Hz, H_2-1″), 5.19 (br t, $J = 6.6$ Hz, H-2″), 1.62 (s, CH_3-4″), 1.76 (s, CH_3-5″), 3.73 (s, 7-OCH_3).

*Garcinone B (**9**)*

Yellow powder, mp 190–192 °C; UV–Vis (EtOH) λ_{max} (log ε) 247 (4.40), 267 (4.40), 339 (4.10), 390 (4.00) nm; FT-IR (KBr) ν_{max} 3480, 1650 cm^{-1}. For ^1H NMR (300 MHz, $CDCl_3$): 13.62 (s, 1-OH), 6.25 (s, H-4), 6.74 (s, H-5), 3.38 (d, $J = 6.6$ Hz, H_2-1′), 5.23 (br t, $J = 6.6$ Hz, H-2″), 1.70 (s, CH_3-4′), 1.77 (s, CH_3-5′), 7.95 (d, $J = 10.2$ Hz, H-1″), 5.75 (d, $J = 10.2$ Hz, H-2″), 1.43 (s, CH_3-4″/5″).

*A mixture of garcinone B (**9**) and macluraxanthone (**10**)*

For ^1H NMR (300 MHz, $CDCl_3$): 13.62 (s, 1-OH), 6.25 (s, H-4), 6.74 (s, H-5), 3.38 (d, $J = 6.6$ Hz, H_2-1′), 5.23 (br t, $J = 6.6$ Hz, H-2″), 1.70 (s, CH_3-4′), 1.77 (s, CH_3-5′), 7.95 (d, $J = 10.2$ Hz, H-1″), 5.75 (d, $J = 10.2$ Hz, H-2″), 1.43 (s, CH_3-4″/5″);13.53 (s, 1-OH), 6.94 (d, $J = 9.0$ Hz, H-7), 7.68 (d, $J = 9.0$ Hz, H-8), 6.76 (d, $J = 9.9$ Hz, H-1′), 5.61 (d, $J = 9.9$ Hz, H-2′), 1.52 (s, CH_3-4′/5′), 6.76 (dd, $J = 17.7$, 10.5 Hz, H-2″), 5.22 (dd, $J = 17.7$, 1.5 Hz, H-3″), 5.05 (dd, $J = 10.5$, 1.5 Hz, H-3″), 1.65 (s, CH_3-4″/5″).

Reagents

Lipopolysaccharide (LPS, from *Escherichia coli*), RPMI-1640 medium, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), L-nitroarginine (L-NA), caffeic acid phenethyl ester (CAPE), indomethacin and phosphate buffer saline (PBS) were purchased from Sigma-Aldrich (Sigma-Aldrich, Missouri, USA). Fetal calf serum (FCS) was bought from Gibco (Invitrogen, California, USA). Penicillin–streptomycin was purchased from Invitrogen (Invitrogen, California, USA). 96-well microplates were obtained from Nunc (Nunc, Birkrød, Denmark).

Other chemicals were from Sigma-Aldrich (Sigma-Aldrich, Missouri, USA).

NO production from RAW264.7 macrophages stimulated by LPS

Inhibitory effect on NO production by murine macrophage-like RAW264.7 cells was evaluated using a modified method from that previously reported (Banskota et al. 2003). Briefly, the RAW264.7 cell line (purchased from cell lines services) was cultured in RPMI medium supplemented with 0.1 % sodium bicarbonate and 2 mM glutamine, penicillin G (100 U/mL), streptomycin (100 μg/mL) and 10 % FCS. The cells were harvested with trypsin–EDTA and diluted to a suspension in a fresh medium. The cells were seeded in 96-well plates with 1×10^5 cells/well and allowed to adhere for 1 h at 37 °C in a humidified atmosphere containing 5 % CO_2. After that the medium was replaced with a fresh medium containing 50 μg/mL of LPS together with the test samples at various concentrations (3–100 μg/mL for crude extract and 3–100 μM for pure compounds) and was then incubated for 24 h. NO production was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent. Cytotoxicity was determined using the MTT colorimetric method. Briefly, after 24 h incubation with the test samples, MTT solution (10 μL, 5 mg/mL in PBS) was added to the wells. After 4 h incubation, the medium was removed, and isopropanol containing 0.04 M HCl was then added to dissolve the formazan production in the cells. The optical density of the formazan solution was measured with a microplate reader at 570 nm. The test compounds were considered to be cytotoxic when the optical density of the sample-treated group was <80 % of that in the control (vehicle-treated) group. L-NA, CAPE and indomethacin were used as positive controls. The stock solution of each test sample was dissolved in DMSO, and the solution was added to the medium RPMI (final DMSO is 1 %). Inhibition (%) was calculated using the following equation and IC_{50} values were determined graphically ($n = 4$):

$$\text{Inhibition } (\%) = \frac{A - B}{A - C} \times 100$$

$A - C$: NO_2^- concentration (μM) [A: LPS (+), sample (−); B: LPS (+), sample (+); C: LPS (−), sample (−)].

Total RNA isolation and RT-PCR

In order to acquire the mechanism of action on cytokine release of compounds **7** and **8** the assays for mRNA expression of iNOS and COX-2 were carried out. The total RNA was isolated from RAW264.7 cells and was harvested after 20 h of incubation with samples in various concentrations (3, 10, 30, 100 μM) using the RNeasy Mini Kit (Qiagen Operon Co. Ltd., USA). The total RNA from each sample was used for cDNA synthesis using first strand cDNA synthesis kit (Rever Tra Ace-α, TOYOBO Co., Ltd., Japan), followed by RT-PCR (Rever Tra Dash, TOYOBO Co., Ltd., Japan). The primers for iNOS and COX-2 were used (forward primer for iNOS: 5′-ATCTGGATCAGGAACCTGAA-3′ and its reverse primer: 5′-CCTTTTTTGCCCCATAGGAA-3′; forward primer for COX-2: 5′-GGAGAGACTATCAAGATAGTGATC-3′ and its reverse primer: 5′-ATGGTCAGTAGACTTTTACAGCTC-3′; forward primer for β-actin (an internal standard): 5′-TGTGATGGTGGGAATGGGTCAG-3′ and reverse primer: 5′-TTTGATGTCACGCACGATTTCC-3′.

The solution for cDNA synthesis consisted of RNA solution 11 μL, 5× RT buffer 4 μL, dNTP mixture (10 mM) 2 μL, RNase inhibitor (10 U/μL) 1 μL, Oligo(dT)20 1 μL and Rever Tra Ace (reverse transcriptase enzyme) 1 μL for a 20 μL reaction. The condition for cDNA synthesis was as follow; 42 °C for 20 min, 99 °C for 5 min and 4 °C for 5 min. After that, 1/10 times (2 μL) of cDNA product was used further for PCR. The PCR mixture consisted of RT reaction mixture (cDNA product) 2 μL; sterilized water 85 μL, 10× PCR buffer 10 μL, forward primer (10 pmol/μL) 1 μL, reverse primer (10 pmol/μL) 1 μL and KOD Dash (polymerase enzyme) 1 μL for final volume of 100 μL. The condition for PCR was as follow; denaturation at 94 °C for 1 min, 98 °C for 30 s, 55 °C for 30 s and 74 °C for 1 min (30 cycles). The PCR products were analyzed in 1.2 % agarose gel electrophoresis and visualized by SYBR safe staining and UV irradiation under a wavelength of 312 nm.

Statistics

For statistical analysis, the values are expressed as mean ± SEM of four determinations. The IC_{50} values were calculated using the microsoft excel programme. The statistical significance was calculated by one-way analysis of variance (ANOVA), followed by Dunnett's test.

Results and discussion

Using Anti-NO assay-guided separation of crude CH_2Cl_2 extract of *C. formosum* ssp. *pruniflorum* roots led to the isolation of ten known xanthones including formoxanthone A (**1**) (Boonsri et al. 2006), pruniflorone K (**2**) (Boonnak et al. 2010a), cochinchinone A (**3**) (Mahabusarakam et al. 2008), pruniflorone L (**4**) (Boonnak et al. 2010a), cochinxanthone E (**5**) (Laphookhieo et al. 2009), 1,7-dihydroxy-8-methoxyxanthone (**6**) (Kijjoa et al. 1998), dulxisxanthone F (**7**) (Deachathai et al. 2005),

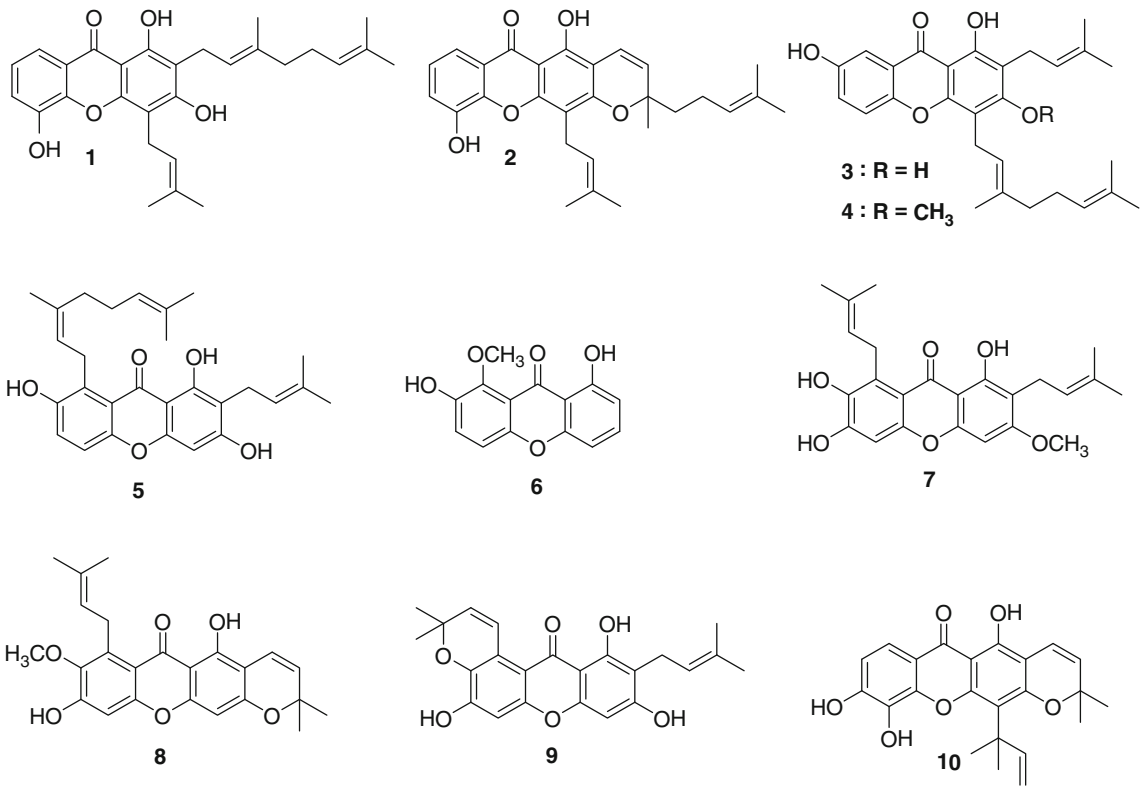

Fig. 1 Structures of compounds **1–10**

Table 1 Nitric oxide inhibitory activity of **1–9** and a mixture of **9** and **10**

Compounds	% Inhibition of various concentrations (μM)[a]						IC_{50} (μM)
	0	1	3	10	30	100	
1	0.0 ± 3.3	–	22.1 ± 1.6*	50.1 ± 2.3**	97.9 ± 0.6[b]**	101.0 ± 3.6[b]**	8.0
2	0.0 ± 3.3	–	–	27.0 ± 2.5*	62.1 ± 3.2**	99.3 ± 2.8**	20.6
3	0.0 ± 2.4	–	–	35.3 ± 0.7**	90.4 ± 1.2[b]**	105.1 ± 3.7[b]**	12.6
4	0.0 ± 3.3	–	–	8.9 ± 2.4	38.9 ± 1.8**	94.9 ± 3.9[b]**	33.1
5	0.0 ± 2.4	–	–	36.9 ± 3.2**	86.3 ± 1.0**	106.5 ± 1.0[b]**	12.8
6	0.0 ± 3.3	–	–	17.7 ± 2.5*	36.2 ± 3.8**	90.8 ± 0.7**	32.8
7	0.0 ± 3.3	12.6 ± 2.5*	50.4 ± 2.5**	78.8 ± 0.4**	93.9 ± 3.0**	101.7 ± 1.2[b]**	3.9
8	0.0 ± 2.4	–	37.8 ± 3.1**	67.1 ± 1.9**	99.1 ± 1.5**	100.9 ± 1.7[b]**	4.3
9	0.0 ± 2.4	–	–	29.9 ± 2.7**	109.0 ± 1.4[b]**	109.6 ± 2.1[b]**	11.8
9 + 10[c]	0.0 ± 2.4	–	22.5 ± 1.8*	104.5 ± 2.0[b]**	106.2 ± 1.8[b]**	106.0 ± 1.4[b]**	12.4
Indomethacin	0.0 ± 4.2	–	16.6 ± 2.9	32.7 ± 2.6**	53.4 ± 3.0**	85.6 ± 1.8**	20.1
L-NA	0.0 ± 5.6	–	15.3 ± 2.8	21.4 ± 2.5	35.6 ± 2.1**	73.2 ± 3.5**	59.0
CAPE	0.0 ± 5.6	–	35.2 ± 3.0*	70.3 ± 2.7**	97.6 ± 2.4[b]**	99.5 ± 2.7[b]**	5.0

Statistical significance * $p < 0.05$; ** $p < 0.01$

[a] Each value represents mean ± SEM of four determinations

[b] Cytotoxic effect was observed

[c] A 1:1 mixture of compounds **9** and **10**

5,9-dihydroxy-8-methoxy-2,2-dimethyl-7-(3-methylbut-2-enyl)-2*H*,6*H*-pyrano-[3,2*b*]-xanthone (**8**) (Sen et al. 1980), garcinone B (**9**) (Sen et al. 1982) and a mixture of garcinone B (**9**) and macruraxanthone (**10**) (Boonnak et al. 2010a) (Fig. 1), which were further evaluated for their inhibitory effects on the LPS-induced NO

production using murine macrophage RAW264.7 cells. From the NO inhibitory activity result in Table 1, it revealed that compounds **7** and **8** exhibited the potent activity against NO release with an IC_{50} value of 3.9 and 4.3 μM, respectively, which are more potent activity than indomethacin (IC_{50} = 20.1 μM) using as the positive control. While compound **1** also exhibited good activity against NO release with an IC_{50} value of 8.0 μM, whereas compounds **3**, **5** and **9** showed moderate activity with an IC_{50} values of 12.6, 12.8 and 11.8 μM, respectively. In order to understand the mechanism of this anti-inflammatory activity, compounds **7** and **8** were further examined for their anti-inflammatory mechanisms against mRNA expressions of COX-2 and iNOS genes by using a semi-quantitative RT-PCR technique. The mechanisms in transcriptional level of **7** was found to down regulate mRNA expressions of iNOS and COX-2 in dose-dependent manners, whereas **8** inhibited only iNOS mRNA expression but did not affect on that of COX-2 gene (Fig. 2). From this result, it could be suggested that compounds **7** and **8** are potent for anti-inflammatory activity by inhibition of NO production.

To conclude, these data suggested that components **1**, **7** and **8** are responsible for NO inhibitory activity with IC_{50} values ranging from 3.9 to 8.0 μM. From the above mention, it could be suggested that *tetra*-oxygenated xanthone skeleton exhibit inhibition of NO production greater than *tri*-oxygenated xanthone skeleton, while a presence of a methoxyl group at C-3 (compound **7**) or C-7 (compound **8**) on the *tetra*-oxygenated isoprenylated-xanthone skeleton are essential for NO inhibitory activity compared to those compound **9**. Compounds **7** and **8** are possible bioactive components responsible for anti-inflammatory activity in the *C. formosum* ssp. *pruniflorum*. This finding could be supported the traditional usage of this plant for the inflammation treatment.

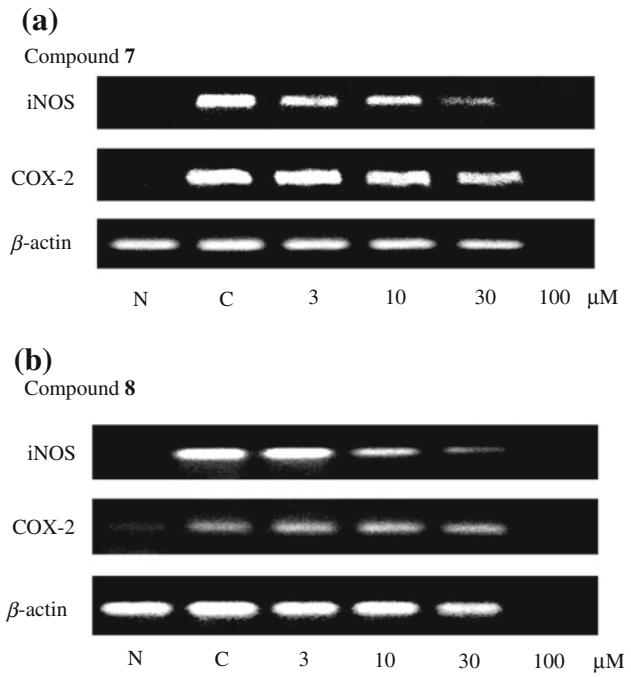

Fig. 2 Effect of **a** compound **7** and **b** compound **8** at various concentrations (0, 3, 10, 30, 100 μM) on mRNA expressions of iNOS and COX-2 using RAW264.7 cells. (N) = LPS (−), sample (−); (C) = LPS (+), sample (−); 3–100 μM = LPS (+), sample (+)

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