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Anti-inflammatory activity of mangostins from *Garcinia mangostana*

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Abstract

The fruit hull of *Garcinia mangostana* Linn (Guttiferae) is used as an anti-inflammatory drug in Southeast Asia. Two xanthones, α- and γ-mangostins, were isolated from the fruit hull of *G. mangostana,* and both significantly inhibited nitric oxide (NO) and PGE_2 production from lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. The IC_{50} values for the inhibition of NO production by α- and γ-mangostins were 12.4 and 10.1 μM, respectively. After iNOS enzyme activity was stimulated by LPS for 12 h, treatment with either α- or γ-mangostin at 5 μg/ml (12.2 and 12.6 μM, respectively) for 24 h did not significantly inhibit NO production. The data show that the inhibitory activities of α- and γ-mangostins are not due to direct inhibition of iNOS enzyme activity. On the other hand, expression of iNOS was inhibited by α- and γ-mangostins in LPS-stimulated RAW 264.7 cells, but not by COX-2. However, the level of PGE_2 production was reduced by the two xanthones. In an *in vivo* study, α-mangostin significantly inhibited mice carrageenan-induced paw edema. In conclusion, α- and γ-mangostins from *G. mangostana* are bioactive substances with anti-inflammatory effects.

Keywords: Inducible nitric oxide synthase; *Garcinia mangostana* Linn; Guttiferae; α- and γ-mangostins; COX-2; RAW 264.7 murine macrophages

1. Introduction

Mangosteen, *Garcinia mangostana* Linn (Guttiferae), is imported from Thailand and cultivated in Taiwan to produce a popular refreshing juicy fruit in the summer. Moreover, the rinds of the fruit have been used as a traditional medicine in Thailand for the treatment of trauma, diarrhea, and skin infections (Nakatani et al., 2002). The xanthones, α- and γ-mangostins, are major bioactive compounds found in the fruit hulls of the mangosteen (Jinsart et al., 1992; Chairungsrilerd et al., 1996a,b,c). The biological activities of α-mangostin have been confirmed to consist of a competitive antagonism of the histamine H1 receptor (Chairungsrilerd et al., 1996a; Iikubo et al., 2002), antibacterial activity against *Helicobacter pylori*, anti-inflammatory activities, inhibition of oxidative damage by human low-density lipoproteins (LDL) (Iikubo et al., 2002), antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (Iinuma et al., 1996), and weak antioxidant activity (Chairungsrilerd et al., 1996a). The other xanthone derivative, γ-mangostin has also been reported to have several pharmacological activities, such as being a potent inhibitor of animal Cdk-activating kinases (Cak), plant Ca^{2+}-dependent protein kinases (CDPK) (Jinsart et al., 1992), and a selective antagonist for $5\text{-}HT_{2A}$ receptors in smooth muscle cells and platelets (Chairungsrilerd et al., 1996b,1998). Moreover, α- and γ-mangostins can inhibit both human immunodeficiency virus (HIV) infection (Chen et al., 1996; Vlietinck et al., 1998), and topoisomerases I and II (Tosa et al., 1997). The mangosteen has long been widely used as an anti-inflammatory, anti-diarrhea, and anti-ulcer agent in Southeast Asia (Lu et al., 1998; Harbborne and Baxter, 1993). However, the actual mechanism of the anti-inflammatory action of xanthones remains unclear. The possibility that xanthones exhibit their biological effects by blocking

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inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression, therefore, was examined in the present study.

Inducible NOS is an important pharmacological target in inflammation and mutagenesis research (Stichtenoth and Frolich, 1998). Therefore, inhibition of NO production by iNOS may have potential therapeutic value when related to inflammation. Furthermore, under inflammatory conditions, macrophages can greatly increase, simultaneously, their production of both NO and the superoxide anion (O^{2-}), which rapidly react with each other to form the peroxynitrite anion ($ONOO^-$), thus playing a role in inflammation and also possibly in the multistage process of carcinogenesis (Xia and Zweier, 1997). The peroxynitrite anion activates the constitutive and inducible forms of cyclooxygenase (COX-1 and COX-2, respectively), which are rate-determining enzymes for prostaglandin biosynthesis during the inflammatory process (Salvemini et al., 1993). On the basis of this evidence, the inhibition of NO production has become a simple approach to examine anti-inflammatory effect.

In the present investigation, NO released from lipopolysaccharide (LPS)-stimulated murine macrophage RAW 264.7 cells was quantitatively analyzed. The effects on iNOS and COX-2 enzyme expression and the level of prostaglandin E_2 (PGE_2) were measured (Wang et al., 2000; Chen et al., 2000), and the effects of the xanthone-derived activities of mangosteen were evaluated by examining NO and PGE_2 production in LPS-activated RAW 264.7 macrophages.

Acute inflammation is a complex process that can be induced by a variety of means. Anti-inflammatory agents exert their effects through a spectrum of different modes of action (Ramprasath et al., 2004). In the screening of new anti-inflammatory compounds, carrageenan-induced edema in the hind paw as an acute inflammation mode is widely employed. Therefore, the carrageenan-induced mice paw edema model was also used to evaluate the anti-inflammatory effects of mangostins in this study.

2. Materials and methods

2.1. General

^1H (500 MHz) and ^{13}C NMR (126 MHz) spectra were measured on a Bruker DRX 500 instrument, and chemical shifts were given in δ (ppm) values. The reversed-phase HPLC was conducted on a Tosoh ODS 80Tm column (4.6 mm i.d. × 250 mm) eluted with 0.05% trifluoroacetic acid-CH_3CN (70: 30). The flow rate was 1.0 mL/min with detection at 280 nm. Column chromatography was carried out using silica gel (Merck). All solvents used for column chromatography were of analytical grade.

2.2. Chemicals and cells

Dimethyl sulfoxide (DMSO), sulindac, *N*-nitro-L-arginine-methyl ester (L-NAME), MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide], trypan blue, LPS (*E. coli* serotype 0127-8B), carrageenan, and other chemicals were purchased from Sigma Chemical (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), antibiotics, L-glutamine, and trypsin-EDTA were purchased from Gibco BRL (Grand Island, NY, USA). The murine macrophage cell line, RAW 264.7, was obtained from American Type Cell Culture (ATCC; Rockville, MD, USA).

2.3. Plant materials

The fruit of *G. mangostana* was purchased in Chiayi, Taiwan. A voucher specimen (NCYU H101) was deposited in the Graduate Institute of Biopharmaceutics of National Chiayi University.

2.4. Isolation

Fresh fruit hulls (1.54 kg) of *G. mangostana* were homogenized with 70% acetone (5 L × 3). The extract was filtered and concentrated in a rotary evaporator to remove the acetone, which produced a reddish-brown extract (149.3 g). The extract (75 g) was dissolved in EtOAc and filtered; the filtrate (17.5 g) was coated on Celite 545, and then subjected to silica gel column chromatography (6.9 cm i.d. × 35 cm) with an *n*-hexane-EtOAc gradient (10:0 → 10:1 → 5:1 → 3:1 → 2:1 → 1:1 → 0:10).

The *n*-hexane-EtOAc (5:1) eluate was rechromatographed through a silica gel column (2 cm i.d. × 40 cm) eluted with a $CHCl_3$–MeOH gradient: from the $CHCl_3$ eluate, to obtain 3.07 g of α-mangostin (**1**), and from the $CHCl_3$–MeOH (10:1) eluate, to obtain 1.74 g of γ-mangostin (**2**). All structures were estimated by EI–MS, and ^1H- and ^{13}C NMR, including 2 D NMR techniques, and also by comparison of those data with authentic compounds. The purity of each compound was determined by reversed-phase HPLC (the retention times of α- and γ-mangostin were 18.2 and 11.6 min, respectively) and both were shown to exceed 98.0% (Fig. 1).

α-Mangostin (**1**) as a fine pale yellow powder; EI-MS *m/z:* 410. ^1H NMR (acetone-d_6, 500 MHz) δ: 1.643, 1.639 (3H each, s, H-5′ and H-5″), 1.77 (3H, s, H-4″), 1.82 (3H, s, H-4′), 3.34 (2H, d, $J = 7.3$ Hz, H-1″), 3.78 (3H, s, $-OCH_3$), 4.12 (2H, d, $J = 6.5$ Hz, H-1′), 5.27 (2H, m, H-2′ and H-2″), 6.38 (1H, s, H-8), 6.80 (1H, s, H-1), 9.42, 9.53 (1H each, brs, C-2-OH and C-7-OH), 13.77 (1H, s, C5-OH). ^{13}C NMR (acetone-d_6, 126 MHz) δ: 17.9 (C-4″), 18.3 (C-4′), 22.0 (C-1″), 25.86, 25.90 (C-5′ and C-5″), 26.9 (C-1′), 61.3 (-O*C*H_3), 93.2 (C-8), 102.7 (C-1), 103.6 (C-5a), 111.1 (C-6), 112.0 (C-4a), 123.5 (C-2″), 124.8 (C-2′), 131.4 (C-3′ and C-3″), 138.1 (C-4), 144.5 (C-3), 155.7 (C-7), 156.2 (C-2), 157.3 (C-1a), 161.7 (C-5), 162.9 (C-8a), 182.8 (C-10).

γ-Mangostin (**2**) as a fine yellow powder; EI-MS *m/z:* 396. ^1H NMR (acetone-d_6, 500 MHz) δ: 1.63 (6 H, s, H-5′ and H-5″), 1.77 (3 H, s, H-4″), 1.83 (3 H, s, H-4′), 3.34 (2 H, d, $J = 7.2$ Hz, H-1″), 4.18 (2 H, d, $J = 6.8$ Hz, H-1′), 5.27 (2 H, m, H-2′ and H-2″), 6.36 (1 H, s, H-8), 6.80 (1 H, s, H-1), 7.60, 9.45, 9.80 (1 H each, brs, C-2-OH, C-3-OH and C-7-OH), 13.91 (1 H, s, C-5-OH). ^{13}C NMR (acetone-d_6, 126 MHz) δ: 17.9 (C-4″), 18.3 (C-4′), 22.0 (C-1″), 25.86, 25.99 (C-5′ and C-5″), 26.4 (C-1′), 92.9 (C-8), 101.1 (C-1), 103.7 (C-5a), 110.8 (C-6), 112.1 (C-4a), 123.6 (C-2″), 124.4 (C-2′), 129.2 (C-4), 131.3 (C-3′ and C-3″), 141.6 (C-3), 152.3 (C-1a), 153.5 (C-2), 155.7 (C-7), 161.7 (C-5), 162.7 (C-8a), 183.2 (C-10).

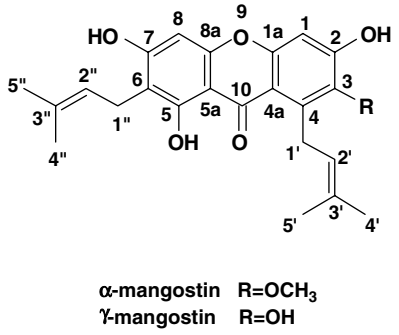

Fig. 1. Structures of α- and γ-mangostins of *Garcinia mangostana*.

2.5. Sample preparation

Test solutions of xanthones (20 mg/ml) were prepared by dissolving each compound in DMSO; they were then stored at 4 °C until use. Serial dilutions of the tested solutions with culture medium were prepared immediately before the in vitro assays were performed.

2.6. NO production by LPS-stimulated RAW 264.7 cells

The murine macrophage cell line, RAW 264.7, was cultivated in DMEM supplemented with 10% FBS at 37 °C in a humidified atmosphere of 5% CO_2. Cells in 96-well plates (0.2 ml, 3×10^5 cells/ml) were treated with LPS (500 ng/ml) and the test compounds. After 18 h, the level of nitrite was measured as described below. The test compounds dissolved in DMSO were diluted with culture medium to concentrations that ranged from 25.0 to 3 μM. The final concentration of DMSO was adjusted to 0.05% (v/v).

2.7. iNOS activity assay

The RAW 264.7 cells were cultured in a 100-mm plate and activated with LPS (1 μg/ml) for 12 h. Cells were collected and washed twice with PBS to remove LPS. RAW 264.7 cell suspensions (0.2 ml) were plated at a concentration of 3×10^5 cells/ml into 96-well plates, and indicated compounds were added. L-NAME as a specific inhibitor of NO synthase enzyme activity was used as a positive control, while 0.5% DMSO was used as a solvent control (Wang et al., 2000). After 12 h, the amount of nitrite was measured by the Griess reaction as described below.

2.8. Cell viability

Mitochondrial respiration, an indicator of cell viability, was assayed by the mitochondrial-dependent reduction of MTT to formazan. Cells in 96-well plates were incubated with MTT (0.25 mg/ml) for 4 h. The cells were solubilized in 0.04 N HCl in isopropanol. The extent of the reduction was measured by the absorbance at 600 nm (Wang et al., 2000).

2.9. Measurement of nitrite formation

Nitrite, as an indicator of NO synthesis, was determined in cell culture supernatants by the Griess reaction (Wang et al., 2000). After incubation of cells for 18 h, the supernatants (0.1 ml) were added to a solution of 0.1 ml Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylene diaminedihydrochloride in 5% H_3PO_4) to form a purple azodye. Using $NaNO_2$ to generate a standard curve, nitrite production was measured by spectrophotometry at 530 nm. Nitrite production was measured by an absorption reading at 530 nm.

2.10. Measurement of PGE_2 production

RAW 264.7 cells were cultured with the test compounds and 500 ng/ml LPS for 18 h. One hundred microliters of supernatant of culture medium was collected for the determination of PGE_2 concentrations with an ELISA kit (Amersham Pharmacia Biotech, UK) (Wang et al., 2000).

2.11. Western blot analysis

RAW 264.7 cells (2 ml, 3×10^5 cells/ml), grown in 6-well plates to confluence, were incubated with or without LPS in the absence or presence of the test compounds for 18 h, respectively. Cells were washed with ice-cold phosphate-buffered saline and stored at –70 °C until further analysis. Protein samples were prepared and resolved by denaturing SDS-PAGE using standard methods (Wang et al., 2000). The proteins were transferred to a nitrocellulose membrane, and Western blotting was performed using a polyclonal rabbit IgG antibody against inducible NO synthase (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-651), a polyclonal goat IgG antibody against COX-2 (sc-1745), and mouse monoclonal IgG_1 antibody against GAPDH (sc-32233). Goat anti-rabbit, anti-mouse, or donkey anti-goat antibodies conjugated to alkaline phosphatase (sc-2007, sc-2022, and sc-2008) and BCIP/NBT (BCIP/NBT, Gibco) were used to visualize protein bands.

2.12. Carrageenan-induced mice paw edema

The mice were divided into three groups ($n = 4$). Acute inflammation was produced by the subplantar administration of 50 μl of 1% carrageenan in normal saline in the right paw of each mouse. The different groups were treated with either α-mangostin (20 mg/kg, *p.o.*), sulindac (20 mg/kg, *p.o.*), or the control vehicle (10% DMSO) administered orally 1 h before the injection of carrageenan. The volume of the paw was measured 1 h before the injection and at 1, 2, 3, 4, 5, and 6 h after the injection of carrageenan. Edema was expressed as the increment in paw thickness due to carrageenan administration (Ramprasath et al., 2004).

2.13. Statistical analysis

Each experiment was performed at least in triplicate. Results are expressed as the mean ± standard deviation (S.D.). The one-way analysis of variance (ANOVA) was used for comparing the paw thickness among the induced, and test groups. p-values < 0.05 were considered significant.

3. Results

3.1. Effects of α- or γ-Mangostin on NO and PGE_2 Produced from LPS-stimulated RAW 264.7 Cells

Xanthones isolated from the70% acetone extracts of mangosteen (see Fig. 1) also inhibited LPS-stimulated NO production and no cytotoxicity to RAW 264.7 cells. The amount of NO production at 3 ~ 25 μM was continuously measured, and the IC_{50} values for the two xanthones were determined. α- or γ-Mangostin dose-dependently reduced the induction of NO products, as shown in Fig. 2, and the IC_{50} values were 12.4 and 10.1 μM, respectively (Table 1). In addition, PGE_2 production by LPS-activated RAW 264.7 cells was measured in the presence of α- or γ-mangostin. In Fig. 3, the data show that these xant-

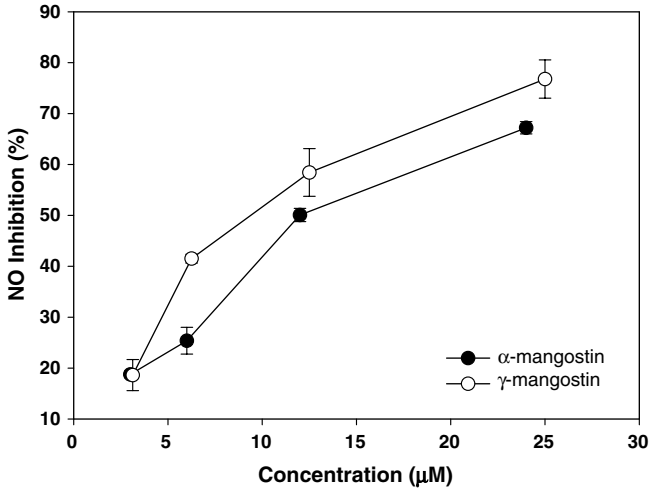

Fig. 2. Nitrite production from LPS-stimulated RAW 264.7 cells co-treated with α- or γ-mangostin. Data are from three separate experiments.

Table 1
The IC_{50} values of α- and γ-mangostins on NO and PGE_2 production inhibition from LPS-stimulated RAW 264.7 cells

Test compounds	IC_{50} (μM)	
	NO production	PGE_2 production
α-Mangostin	12.4	11.08
γ-Mangostin	10.1	4.50

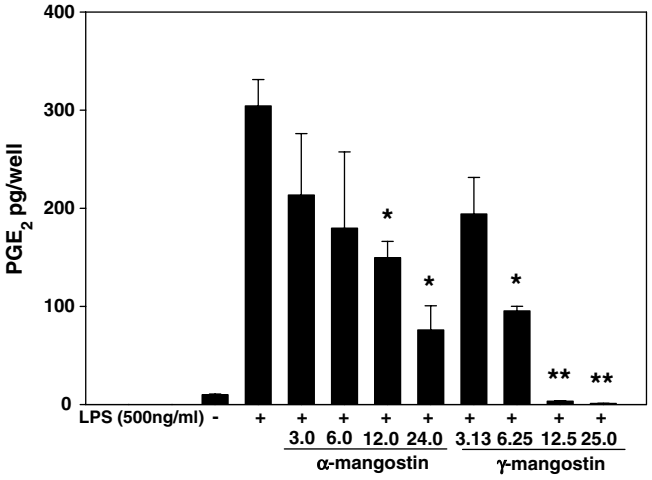

Fig. 3. PGE_2 production from LPS-stimulated RAW 264.7 cells co-treated with α- or γ-mangostin. Statistical analysis was done using the Student's *t*-test. $^{*}p < 0.01$; $^{**}p < 0.001$, significantly different from the 0.05% DMSO-treated group. Data are from three separate experiments.

hones also significantly reduced PGE_2 production in a dose-dependent manner and that γ-mangostin had a stronger efficacy than α-mangostin.

3.2. Effects of α- or γ-Mangostin on iNOS and COX Enzyme Expressions

The effects of the test compounds on the induction of iNOS and COX enzyme expressions were checked using a Western blot technique. As shown in Fig. 4, α- or γ-mangostin concentration-dependently reduced the induction of iNOS at 3–25 μM, and the inhibitive effects of γ-mangostin were also stronger than these of α-mangostin. The two xanthones significantly inhibited the expression of iNOS, but not COX-2, as shown in Fig. 4.

3.3. Effects of α- or γ-Mangostin on iNOS enzyme activity

It is unknown whether the reduction in nitrite accumulation by α- or γ-mangostin is a result of the inhibition of iNOS expression or inhibition of its enzymatic activity. The effects of α- or γ-mangostin were compared with those of L-NAME, a specific inhibitor of NO synthase enzyme activity. RAW 264.7 cells were activated by LPS (1 μg/ml) for 12 h, after which the medium was replaced with fresh medium containing the test compounds. α- or γ-

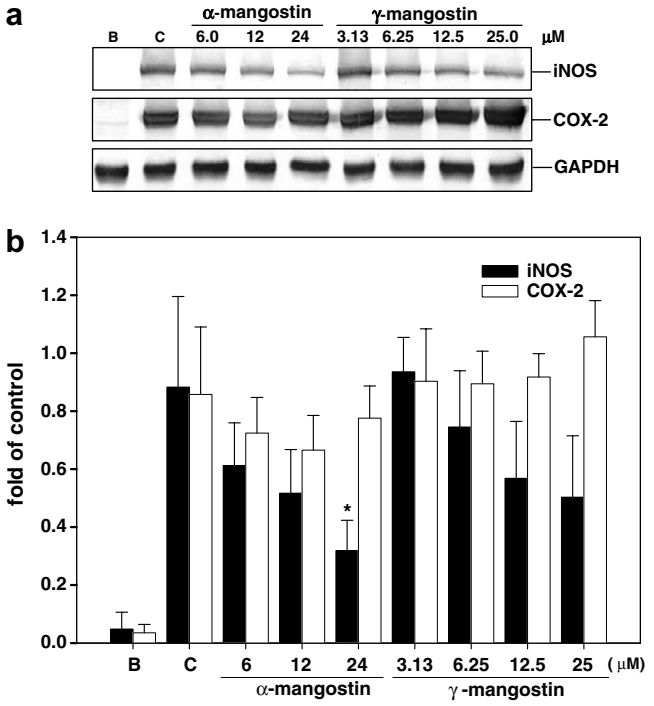

Fig. 4. iNOS and COX-2expression from LPS-stimulated RAW 264.7 cells co-treated with α- or γ-mangostin. (a) Protein levels of iNOS and COX-2, determined by Western blot analysis. Equal loading was confirmed by stripping the blot and reprobing it for GAPDH. Data are from three separate experiments, one of which is illustrated. (b) Histogram representing the relative density of the Western blot bands normalized to GAPDH. B indicates no treatment with LPS, C indicates the 0.05% DMSO-treated group in the presence of LPS, * denotes a significant difference at $p < 0.05$.

Table 2
Effects of α- or γ-mangostin on iNOS enzyme activity after LPS-activated RAW 264.7 cells

Test Compounds	NO production inhibition (%)
DMSO, 0.025%	8.58 ± 1.1
α-Mangostin, 12.2 μM	4.24 ± 1.8
γ-Mangostin, 12.6 μM	28.69 ± 0.8
L-NAME, 200.0 μM	55.94 ± 1.2

LPS (1 μg/ml) pretreatment of RAW 264.7 cells for 12 h and then iNOS was activated. The active RAW 264.7 cells were replaced with fresh medium containing the test compounds.
Results are expressed as the mean ± S.D. of three experiments.
DMSO (0.025%) was used as the solvent in this experiment.
L-NAME (200.0 μM), an NOS activity inhibitor, was used as a positive control.

Mangostin (both at 5.0 μg/ml), or the control solvent (0.25% DMSO) weakly inhibited iNOS activity in activated RAW 264.7 macrophages. In contrast, L-NAME significantly inhibited nitrite accumulation by more than 50% at 200 μM (Table 2). According to the above results, we suggest that neither α- nor γ-mangostin exhibits a direct inhibitory effect on the enzymatic activity of inducible NO synthase.

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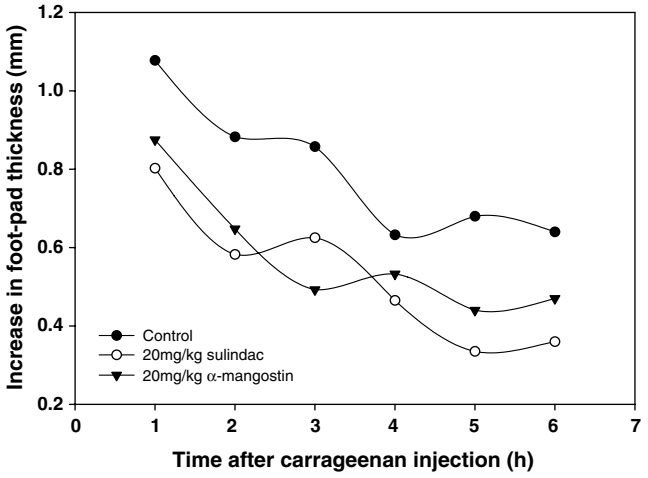

Fig. 5. Anti-inflammatory effects of α-mangostin and sulindac on carrageenan-induced paw edema in mice. Control: solvent control (10% DMSO). Values are expressed as the mean of four animals. Sulindac was used as a reference drug. Both α-mangostin and sulindac treatment showed significant difference when compared with control group (α-mangostin vs. control, $p = 0.001$; sulindac vs. control, $p = 0.006$).

3.4. Effects of α-mangostin on carrageenan-induced paw edema in mice

The anti-inflammatory effects of α- and γ-mangostins were evaluated by carrageenan-induced paw edema in mice that was used as an acute model of inflammation. The *in vivo* data of the experiment have been analyzed by ANOVA. Both α-mangostin and sulindac treatment showed significant difference on paw edema inhibition when compared with control group (α-mangostin vs. control, $p = 0.001$; sulindac vs. control, $p = 0.006$). α-Mangostin and sulindac exhibited a potent inhibition on paw edema at 3 h and 5 h, respectively (Fig. 5). Therefore, we suggested the on-set time of paw edema inhibition from the α-mangostin was more quickly than that of sulindac. However, γ-mangostin did not significant inhibit the paw edema in mice (data not shown). The data demonstrated that α-mangostin has more anti-inflammatory activity than γ-mangostin *in vivo*.

4. Discussion

The genus *Garcinia* (Guttiferae) is a group of well known fruit trees in Malaysia. The fruit of many species are edible and serve as a substitute for tamarinds in curries. Many species produce a yellow resin which is used in making varnishes and treating wounds. Some species have been shown to exhibit significant antimicrobial and pharmacological activities (Valdir et al., 2000). The mangosteen tree, *G. mangostana* is one of these, and its fruit is rich in a variety of oxygenated and prenylated xanthones (Valdir et al., 2000; Suksamrarn et al., 2002; Nilar, 2002). Moreover, the fruit hulls of *G. mangostana* also contain abundant xanthones such as 8-desoxygartanin, and α-, β-, and γ-mangostins (Chairungsrilerd et al., 1996b; Huang et al., 2001; Gopalakrishnan et al., 1997). These xanthones have demonstrated antibacterial (Iinuma et al., 1996), antifungal (Gopalakrishnan et al., 1997), antitumor-promotion (Suksamrarn et al., 2002), and cytotoxic characteristics in HL-60 cells (Katsumoto et al., 2003; Matsumoto et al., 2004). In this study, α- and γ-mangostins were isolated from the fruit hulls of *G. mangostana*, and their anti-inflammatory effects were investigated. The results showed that α- and γ-mangostins could significantly inhibit NO and PGE_2 production and iNOS expression by LPS-stimulated RAW 264.7 cells, with γ-mangostin showing stronger inhibitory effects than α-mangostin. However, iNOS activity and COX-2 expression were not inhibited by α-mangostin or γ-mangostin. We suggest that the two mangostins decrease PGE_2 levels through inhibition of COX-2 activity and NO production. As previous reports demonstrated, mangostins can inhibit COX-2 activity in C6 rat glioma cells (Nakatani et al., 2002, 2004). Furthermore, NO activates the constitutive and inducible forms of cyclooxygenase (COX-1 and COX-2, respectively), which are rate-determining enzymes for PGE_2 biosynthesis during the inflammatory process (Salvemini et al., 1993).

The most widely used primary test for screening of anti-inflammatory agents is carrageenan-induced edema in the mice hindpaw. The development of edema in the paw of the mice after injection of carrageenan was described by Vingar et al. (Vinegar et al., 1969) as a biphasic event. The initial phase observed during the first hour was attributed to a release of histamine and serotonin (Kumar et al., 2004); the second phase was due to a release of prostaglandin-like substances (Kumar et al., 2004). In the present results, suppressive activity by α-mangostin was exhibited in both phases; however a significant inhibitory effect was seen after treatment for 3 h. We suggest that α-mangostin shows a more potent inhibition of PGE_2 release than either histamine or serotonin. On the other hand, γ-mangostin inhibited mice carrageenan-induced paw edema, which has also been previously reported (Nakatani et al., 2004). Therefore, the above results demonstrate that α- and γ-mangostins from the fruit hulls of *G. mangostana* are anti-inflammatory substances, and can serve as lead compounds in the development of anti-inflammatory drugs.

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