European Journal of Medicinal Chemistry 48 (2012) 371–378

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Short communication

A concise synthesis of viscolin, and its anti-inflammatory effects through the suppression of iNOS, COX-2, ERK phosphorylation and proinflammatory cytokines expressions

Guan-Jhong Huang [a,1], M. Vijaya Bhaskar Reddy [b,1], Ping-Chung Kuo [c], Chieh-Hung Huang [b], Hung-Cheng Shih [b], E-Jian Lee [d], Mei-Lin Yang [b], Yann-Lii Leu [e,1], Tian-Shung Wu [b,f,g,*]

[a] School of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, College of Pharmacy, China Medical University, Taichung 404, Taiwan, ROC
[b] Department of Chemistry, National Cheng Kung University, Tainan 701, Taiwan, ROC
[c] Department of Biotechnology, National Formosa University, Yunlin 632, Taiwan, ROC
[d] Neurophysiology Laboratory, Neurosurgical Service, Departments of Surgery and Anesthesiology, and Institute of Biomedical Engineering, National Cheng Kung University Medical Center and Medical School, Tainan 701, Taiwan, ROC
[e] Graduate Institute of Natural Products, Chang Gung University, Taoyuan 333, Taiwan, ROC
[f] Department of Pharmacy, China Medical University, Taichung 404, Taiwan, ROC
[g] Chinese Medicine Research and Development Center, China Medical University and Hospital, Taichung 404, Taiwan, ROC

ARTICLE INFO

Article history:
Received 9 September 2011
Received in revised form
14 November 2011
Accepted 4 December 2011
Available online 9 December 2011

Keywords:
Viscolin
Anti-Inflammatory
Macrophages
*i*NOS
COX-2
λ-Carrageenin

ABSTRACT

In the present report, a concise synthesis of viscolin (**1**) has been achieved. The anti-inflammatory effect of viscolin was investigated *in vitro* and *in vivo*. Viscolin blocked the expression of *i*NOS and COX-2, and it also inhibited the ERK for the activation of NF-κB in LPS-stimulated RAW 264.7 macrophages. Western blotting and immunohistochemical analysis revealed that viscolin decreased Carr-induced *i*NOS and COX-2 expressions. These results could help to deduce the anti-inflammatory mechanisms.

1. Introduction

Inflammation leads to the signaling proteins in affected cells and tissues. Inducible nitric oxide synthase (iNOS), catalyzes the formation of nitric oxide (NO) from L-arginine [1]. Excess production of NO has shown to be associated with a number of chronic diseases, including asthma, rheumatoid arthritis, inflammatory bowel disease, atherosclerosis, Alzheimer's disease, and various human cancers [2]. Thus, inhibition of NO synthesis and PGE2 production stands as an important therapeutic goal. Viscolin **1** is a naturally occurring 1,3-diarylpropane, identified from *Viscum coloratum* (Loranthaceae) [3], a hemiparasite herb used in traditional Chinese medicine for the treatment of a number of ailments such as hemorrhage, gout, heart disease, epilepsy, arthritis and hypertension [4,5]. The structural novelty of viscolin **1**, were found to exhibit potent and selective inhibition of superoxide anion generation ($O_2\bullet^-$) and elastase release induced with *N*-formyl-methionyl-leucephenyl alanine combined with cytochalasin B (fMLP/CB) in human neutrophils with IC_{50} values of 0.58 ± 0.03 and 4.93 ± 0.54 μg/mL, respectively [6]. Viscolin does not show structural similarity to any known phosphodiesterase inhibitor and thus provides a new chemical skeleton in the development of PDE inhibitors [6]. In addition, viscolin exhibits leukocyte inhibitory activity by suppressing free radicals, possibly through modulation of PKC activity and calcium mobilization, and NO production with moderate free radical-scavenging effects that give viscolin the potential to be anti-inflammatory agent for the treatment of oxidative stress-induced diseases [6].

* Corresponding author. Department of Chemistry, National Cheng Kung University, Graduate Institute of Natural Products, 1, Ta-Shiueh Rd.Tainan 701, Taiwan, ROC. Tel.: +886 6 2747538; fax: +886 2 2740552.
E-mail address: tswu@mail.ncku.edu.tw (T.-S. Wu).
[1] Among authors provided equal contributions to this work.

0223-5234/$ – see front matter © 2011 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2011.12.008

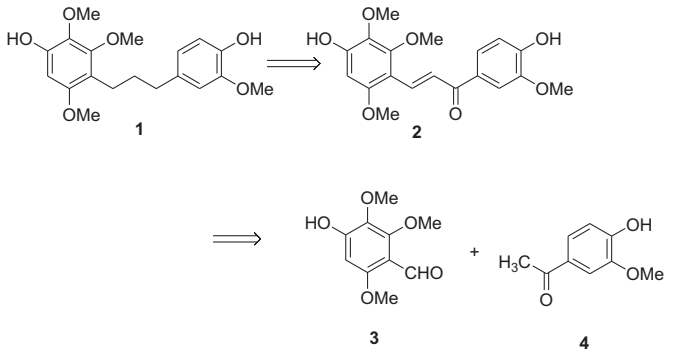


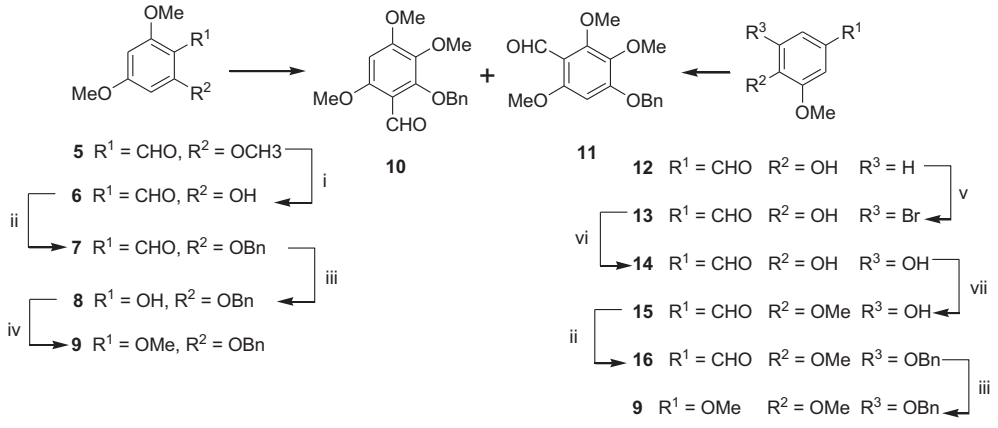
Fig. 1. Retrosynthetic analysis of viscolin 1.

It has been reported that inflammatory effect induced by Carr could be associated with free radicals in the previous literature [7]. To explore the *in vitro* and *in vivo* anti-inflammatory effects of viscolin, in the present study we reported a concise synthesis of viscolin by a strategy distinctly different from that of our previous report [8]. With the assistance of this concise synthetic method, the anti-inflammatory effects of viscolin on LPS-induced in RAW 264.7 cells and Carr-induced on paw edema in mice could be examined. The levels of *i*NOS and COX-2 in either RAW 264.7 cell or paw edema were also detected and the activities of CAT, SOD, and GPx at the 5th hr after Carr injection were also investigated to elucidate the relationship between the anti-inflammatory mechanism of viscolin and antioxidant enzymes.

2. Chemistry

The retrosynthetic analysis of viscolin **1** was illustrated in Fig. 1. It displayed that hydrogenation of the intermediate **2** which was prepared through the aldol condensation of the key precursors **3** and **4** could approach the target compound **1**. Thus our study commenced with the preparation of the substrate **9** for the formylation reaction to the analog **10** of the precursors **3** as shown in Fig. 2. The tetra-oxygenated benzenoid **9** could be synthesized in two pathways. In the first route, the commercially available 2,4,6-trimethoxybenzaldehyde **5** was regioselectively mono-deme thylated induced by BBr_3 as a Lewis acid in *ortho*-position with the assistance of the *ortho*-directing effect of the acyl substituent [9,10]. The resulting 2-hydroxy-4,6-dimethoxybenzldehyde **6** was again protected with benzyl group in basic dimethylformamide solution to afford **7**. Baeyer-Villiger oxidation of **7** with *m*-CPBA in CH_2Cl_2 gave the ester [11], which was further hydrolyzed with aqueous NaOH and methanol to furnish phenol **8**. Finally the phenol group in **8** was methylated with dimethyl sulfate to yield colorless oil **9**. In another pathway, production of the substrate **9** started from the commercially available vanillin **12**. Treatment of **12** with Br_2 in glacial acetic acid yielded 5-bromovanillin **13** in high yields [12], which was converted to 5-hydroxyvanillin **14**, by reflux with copper powder and aqueous NaOH solution [13]. Methylation of **14** with 1.1 equivalent of dimethyl sulfate gave the desired benzaldehyde **15** in good yields and only trace amount of the regioisomeric product, syringaldehyde were detected by TLC. This by-product could be easily removed from the desired product by over methylation of syringaldehyde to 3,4,5-trimethoxybenzal dehyde, and alkaline extraction followed by acidification of the reaction mixture. Compound **15** was protected with benzyl group in basic acetone solution to afford **16** and further Baeyer-Villiger oxidation of **16** with *m*-CPBA in CH_2Cl_2 gave the ester, which was hydrolyzed in basic solution to afford phenol **17**. This reaction crude was directly subjected into methylation with dimethyl sulfate to yield **9** in 30%. Both the routes for the preparation of substrate **9** provided the desired products in good yields ranging from 12 to 27%. Although it offered the substrate **9** with the lower yield in the second synthetic route, the starting material **12** was cheaper and more economic for the large scale production of the final products viscolin (**1**).

Vilsmeier reaction of **9** with dimethylformamide and phosphorus oxychloride gave the mixtures of two isomeric aldehydes **10** and **11** purified by silica gel column chromatography in 51% and 16% yields, respectively. In addition, compound **18** was protected with benzyl group in basic dimethylformamide solution to afford **19**. Coupling of aldehydes **10** and 4-*O*-benzyl-3-methoxyacetophenone **19** was achieved with the aid of Aldol condensation in basic condition to afford chalcone **20** (Fig. 3). This could be further reduced by hydrogenation with the catalyst of 10% Pd/C in ethyl acetate-methanol (9:1) solution to produce viscolin **1** in fewer steps and better yields (7 steps, 8%), compared with the previous report (15 steps, 6%) [8].



Reagents and conditions: (i) BBr_3 in 1.0M CH_2Cl_2, -78^{O}C, 1.5 h. **(ii)** BnCl, DMF, K_2CO_3, reflux, 6h. **(iii)** (1) *m*-CPBA, CH_2Cl_2, room temp.(2) NaOH-MeOH, room temp. 3 h. **(iv)** $(CH_3)_2SO_4$, K_2CO_3, reflux, 4h. **(v)** Br_2/acetic acid, room temp. **(vi)** NaOH soulution in Cu powder, reflux. **(vii)** dimethyl sulphate, Na_2CO_3, reflux.

Fig. 2. Preparation of the precursors 9 and 10.

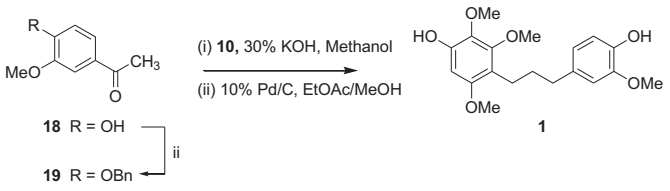


Fig. 3. Synthesis of viscolin 1.

3. Results and discussion

3.1. Effects of viscolin on LPS-induced NO, TNF-α, and PGE_2 production in RAW 264.7 macrophages

The RAW 264.7 cells were incubated for 24 h with 100 ng/mL of LPS (lipopolysaccharide) in the absence or presence of viscolin (0, 5, 10, and 20 μM). Viscolin was added 1 h before incubation with LPS. Cell viability assay was performed using MTT assay. Nitrite concentration in the medium was determined using Griess reagent. TNF-α and PGE_2 levels in the medium were determined using ELISA kit. Cells cultured with or without viscolin did not change cell viability significantly (Fig. S1A). NO plays a role as neurotransmitter, vasodilator, and immune regulator in a variety of tissues at physiological concentration. High levels of NO produced by *i*NOS have been defined as a cytotoxic molecule in inflammation [1]. In the present study, effects of viscolin on LPS-induced NO production in RAW 264.7 macrophages were investigated. Nitrite accumulated in the culture medium was estimated by the Griess reaction as an index for NO release from the cells. Viscolin did not interfere with the reaction between nitrite and Griess reagents at 20 μM (data not shown). Unstimulated macrophages after 24 h of incubation in culture medium produced background levels of nitrite. When RAW 264.7 macrophages were treated with different concentrations of viscolin (0, 5, 10, and 20 μM) together with LPS (100 ng/mL) for 24 h, a significant concentration-dependent inhibition of nitrite production was detected (Fig. S1B). There was either a significant decrease in the nitrite production of group treated with 5 μM viscolin ($p < 0.05$), or highly significant decrease of groups treated respectively with 10 and 20 μM of viscolin when compared with the LPS-alone group ($p < 0.01$ or $p < 0.001$). The IC_{50} value for inhibition of nitrite production of viscolin was 17.80 ± 1.52 μM.

TNF-α plays an important role in the promotion of the inflammatory response, which in turn causes many clinical problems associated with autoimmune disorders, such as rheumatoid arthritis, Crohn's disease, psoriasis, and asthma [14]. After treatment with LPS (100 ng/mL) for 24 h, the TNF-α concentration increased in the medium. When RAW 264.7 macrophages were treated with different concentrations of viscolin (0, 5, 10, and 20 μM) together with LPS (100 ng/mL) for 24 h, a significant concentration-dependent inhibition of TNF-α level was detected (Fig. S1C). There was either a significant decrease in the TNF-α level of group treated with 5 μM viscolin ($p < 0.05$), or highly significant decrease of groups treated respectively with 10 and 20 μM of viscolin when compared with the LPS-alone group ($p < 0.01$ or $p < 0.001$). The IC_{50} value for inhibition of TNF-α level of viscolin was 17.32 ± 0.08 μM.

An increase of PGE_2 production has been demonstrated by LPS treatment. After treatment with LPS (100 ng/mL) for 24 h, the amount of PGE_2 elevated clearly in the medium, and viscolin at 10 or 20 μM in the presence of LPS was able to significantly suppress the LPS-induced production of PGE_2 in RAW 264.7 macrophages when compared with the LPS-alone group ($p < 0.01$ or $p < 0.001$) (Fig. S1D). The IC_{50} value for inhibition of PGE_2 level of viscolin was about 14.37 ± 0.12 μM.

3.2. Effects of viscolin on the LPS-stimulated activation of iNOS, COX-2, and mitogen-activated protein kinases (MAPKs)

The pathology of inflammation is initiated by complex processes triggered by microbial pathogens such as LPS, which is a prototypical endotoxin. LPS can directly activate macrophages, which trigger the production of inflammatory mediators, such as NO, TNF-α and leukotrienes [15]. The pharmacological reduction of LPS-inducible inflammatory mediators (for example NO and TNF-α) is regarded as one of the essential conditions to alleviate a variety of disorders caused by activation of macrophages. Thus, RAW 264.7 macrophages provide us with an excellent model for anti-inflammatory drug screening and for subsequently evaluating the inhibitors of the pathways that lead to the induction of proinflammatory enzymes and to the production of proinflammatory cytokines [16]. In order to investigate whether the inhibitions of NO, TNF-α, and PGE_2 production were due to the decreased *i*NOS and COX-2 protein levels, the effects of viscolin on *i*NOS and COX-2 protein expression were studied by immunoblot. The results exhibited that incubation with viscolin (0, 5, 10, and 20 μM) in the presence of LPS (100 ng/mL) for 24 h inhibit *i*NOS and COX-2 proteins expression in mouse macrophage RAW 264.7 cells in a dose-dependent manner (Fig. S2A). The detection of β-actin was also performed in the same blot as an internal control. The intensity of protein bands were analyzed using Kodak Quantity software in three independent experiments and showed an average of 83.8 and 70.6% down-regulation of *i*NOS and COX-2 proteins, respectively, after treatment with viscolin at 20 μM compared with the LPS-alone (Fig. S2B). These *in vitro* data showed that viscolin suppressed LPS-induced productions of NO, TNF-α, and PGE_2, which are the expression products of inflammatory protein such as *i*NOS and COX-2.

MAPKs play critical roles in the regulation of cell growth and differentiation, and control cellular responses to cytokines and stresses. In particular, ERK, p38, and JNK are known to be important for the activation of NF-κB [17]. To explore whether the inhibition of NF-κB activation by viscolin is mediated through the MAPK pathway, MAPK phosphorylation was examined by Western blot in RAW 264.7 cells pretreated with viscolin and then with LPS. As shown in Fig. S3, we showed that ERK, JNK, and p38 were phosphorylated with LPS stimulation. Furthermore, phosphorylation of ERK was inhibited by viscolin at 30 min of LPS stimulation, whereas there was no effect of viscolin on p-p38 or p-JNK. MAPKs are also likely targets for the development of novel anti-inflammatory drugs; however, signaling from MAPKs to transcription factors mediating *i*NOS and COX-2 expression is not fully understood.

3.3. Effects of viscolin on Carr-induced mice paw edema

Since viscolin effectively inhibited *i*NOS and COX-2 expressions in macrophages, studies were extended to determine whether viscolin affected acute phase inflammation in animal models. In the present study, the Carr-induced edema model was performed due to its widely adaptation for screening the effects of anti-inflammatory drugs. Carr-induced paw edema is shown in Fig. S4A. Viscolin (8 mg/kg) significantly inhibited ($p < 0.001$) the development of paw edema-induced by Carr after 4th and 5th hr of treatment, comparable with the reference compound Indo (10 mg/kg). Similarly, the MDA level increased significantly in the edema paw at 5th hr after Carr injection ($p < 0.001$). However, the MDA level was decreased significantly by treatment with viscolin (8 mg/kg) ($p < 0.001$) as well as 10 mg/kg Indo (Fig. S4B). In addition, the NO and TNF-α levels increased significantly in the edema serum at 5th hr after Carr injection ($p < 0.001$). Viscolin (8 mg/kg)

significantly decreased the serum NO and TNF-α levels ($p < 0.001$). The inhibitory potency was similar to that of Indo (10 mg/kg) at 5th hr after induction (Fig. S4C and D).

The Carr-induced rat paw edema is a suitable test for evaluating anti-inflammatory drugs and has frequently been used to assess the anti-edematous effect of natural products [18]. The degree of swelling of the Carr-injected paws was maximal 3rd hr after injection. Statistical analysis revealed that viscolin and Indo significantly inhibited the development of edema 4 h after treatment ($p < 0.001$). They both showed anti-inflammatory effects in Carr-induced mice edema paw. It is well known that the third phase of the edema-induced by Carr, in which the edema reaches its highest volume, is characterized by the presence of prostaglandins and other compounds of slow reaction found that the injection of Carr into the rat paw induces the liberation of bradykinin, which later induces the biosynthesis of prostaglandin and other autacoids, which are responsible for the formation of the inflammatory exudates [19]. The proinflammatory cytokines such as TNF-α are small secreted proteins, which mediate and regulate immunity and inflammation. The production of TNF-α is crucial for the synergistic induction of NO synthesis in IFN-γ and/or LPS-stimulated macrophages. TNF-α induces a number of physiological effects including septic shock, inflammation, and cytotoxicity [20]. Also, TNF-α is a mediator of Carr-induced inflammatory incapacitation, and is able to induce the further release of kinins and leukotrienes, which is suggested to be an important role in the maintenance of long-lasting nociceptive response [21]. In the above results, viscolin significantly decreased the TNF-α level in serum after Carr injection by treatment with 2, 4, and 8 mg/kg, respectively. It suggested that anti-inflammatory effects of viscolin were resulted from the inhibition of the proinflammatory cytokines in the Carr-induced paw edema.

3.4. Effects of viscolin on activities of antioxidant enzymes

The acute inflammatory response is associated with the production of reactive oxygen species (ROS), which have been proposed to mediate cell damage in the paw tissue. At 5th hr after the intrapaw injection of Carr, paw tissues were analyzed for the biochemical parameters, such as CAT, SOD, and GPx activities. CAT, SOD, and GPx activities in paw tissue were decreased significantly by Carr administration. CAT, SOD, and GPx activity were increased significantly after treated with 8 mg/kg viscolin and 10 mg/kg Indo ($p < 0.01$) (Table 1). This local acute inflammation model induces a biphasic edema consisting of an early phase (up to 2 h) followed by a more sustained late phase (2–6 h). The early phase of Carr edema is related to the production of immediate inflammation mediators such as histamine, bradykinin, leukotrienes, platelet-activating factor and cyclooxygenase products in the inflamed tissue. The late phase is related to neutrophil infiltration and the production of ROS. In a number of pathophysiological conditions associated with inflammation or oxidant stress, these ROS have been proposed to mediate cell damage via a number of independent mechanisms including the initiation of lipid peroxidation, the inactivation of a variety of antioxidant enzymes and depletion of glutathione. Giving the importance of the oxidative status in the formation of edema, the anti-inflammatory effect exhibited by drug in this model might be related to its antioxidant properties [22]. In our study, there is a significantly increment in CAT, SOD, and GPx activities with viscolin treatment. Furthermore, significant decreases in MDA level with viscolin treatment were also found. These results indicated that the suppression of MDA production is probably due to the enhancements of CAT, SOD, and GPx activities.

Table 1
Effects of viscolin and indomethacin (Indo) on changes in CAT, SOD, and GPx activities in Carr-induced paw edema (5th hr) in mice.

Groups	CAT (U/mg protein)	SOD (U/mg protein)	GPx (U/mg protein)
Control	5.26 ± 0.14	4.31 ± 0.16	12.96 ± 0.12
Carr	3.54 ± 0.15[a]	1.65 ± 0.13[a]	7.21 ± 0.07[a]
Carr + Indo (10 mg/kg)	4.65 ± 0.17[c]	3.85 ± 0.24[c]	10.45 ± 0.09[c]
Carr + viscolin (2 mg/kg)	3.92 ± 0.13[b]	2.45 ± 0.12[b]	8.64 ± 0.05[b]
Carr + viscolin (4 mg/kg)	4.28 ± 0.11[c]	3.46 ± 0.11[c]	10.97 ± 0.11[c]
Carr + viscolin (8 mg/kg)	4.98 ± 0.23[a]	4.08 ± 0.19[a]	11.54 ± 0.16[a]

Each value represents as mean ± S.E.M.
[a] $p < 0.001$ as compared with the control group.
[b] $p < 0.05$ and.
[c] $p < 0.01$ as compared with the Carr (λ-carrageenan) group (one-way ANOVA followed by Scheffe's multiple range test).

3.5. Effects of viscolin on Carr-induced iNOS and COX-2 protein expressions in mice paw edema

To investigate whether the inhibition of NO production was due to a decreased *i*NOS and COX-2 protein levels, the effects of viscolin on *i*NOS and COX-2 proteins expression were studied by Western blot. The results showed that injection of viscolin (8 mg/kg) on Carr-induced for 5th hr inhibited *i*NOS and COX-2 proteins expression in mouse paw edema (Fig. S5A). The intensity of protein bands were analyzed using Kodak Quantity software in three independent experiments and showed an average of 63.6% and 76.2% down-regulation of *i*NOS and COX-2 proteins, respectively, after the treatment with viscolin compared with the Carr-induced alone (Fig. S5B). In addition, the protein expressions displayed an average of 69.1 and 73.1% down-regulation of *i*NOS and COX-2 protein after the treatment with Indo at 10.0 mg/kg compared with the Carr-induced alone. The down-regulation of *i*NOS and COX-2 activity of viscolin (8 mg/kg) exhibited as well as Indo (10.0 mg/kg) did.

3.6. Histological examination

Paw biopsies of the control mice displayed marked cellular infiltration in the connective tissue. The infiltrates accumulated in collagen fibers and intercellular spaces. Paw biopsies of mice treated with viscolin (8 mg/kg) showed a reduction in inflammatory responses induced by Carr. Histologically, inflammatory cells were reduced in number and confined to the surroundings of the vascular areas. Intercellular spaces did not show any cellular infiltrations. Collagen fibers were regular in shape and exhibited a reduction in intercellular spaces. Moreover, the hypodermis connective tissues were not damaged (Fig. S6A). Neutrophils were increased with Carr treatment (Fig. S6B). Indomethacin and viscolin (8 mg/kg) could decrease the neutrophils numbers as compared to the Carr-treated group (Fig. S6C and D, respectively). At 5th h after intraplantar Carr injection, numerous *i*NOS and COX-2 immunoreactive cells were observed in the brown site of paw tissue (Fig. S6E–H). Administration of indomethacin and viscolin (8 mg/kg) 30 min prior to the Carr injection markedly reduced the increase in *i*NOS and COX-2 immunoreactive cells in paws (Fig. S6I–L).

4. Conclusion

In the present study, a concise synthesis of viscolin has been achieved to construct the 1,3-diarylpropane skeleton and maintain the hydroxyl groups at *para* position in both rings. In addition, we demonstrated anti-inflammatory activities of viscolin in

both *in vitro* and *in vivo* experimental systems, using LPS-stimulated RAW 264.7 macrophages and a mouse model of topical inflammation respectively. The anti-inflammatory mechanism of viscolin may be related to *i*NOS, COX-2, and it is associated with the increase in the activities of antioxidant enzymes (CAT, SOD, and GPx). Dual inhibitory activities against *i*NOS as shown in *in vitro* assays appear to confer on viscolin a potent *in vivo* efficacy in mouse, Carr-induced, paw edema, comparable with a potent and well known COX inhibitor, Indo, suggesting that viscolin may be used as a pharmacological agent in the prevention or treatment of disease in which free radical formation in a pathogenic factor.

5. Experimental section

5.1. General

Unless stated otherwise, the chemicals were acquired from commercial sources and used without further purification. Melting points were measured on a Yanaco MP-S3 micro melting point apparatus and are uncorrected. IR spectra were determined on a Shimadzu FT-IR Prestige 21 spectrophotometer. ^{1}H and ^{13}CNMR spectra were recorded on a Bruker Avance 300 spectrometer, using tetramethylsilane (TMS) as internal standard; all chemical shifts are reported in parts per million (ppm, δ). EIMS and HREIMS spectra were obtained on a VG 70-250S mass spectrometer. Column chromatography was performed on silica gel (70–230 mesh, 230–400 mesh). TLC was conducted on pre-coated Kieselgel 60 F254 plates (Merck), and the spots were examined under UV light and revealed by a sulfuric acid-anisaldehyde spray.

5.2. Chemistry

5.2.1. 2-Hydroxy-4,6-dimethoxybenzaldehyde (**6**)

To a solution of 2,4,6-trimethoxybenzaldehyde (**5**) (10.0 g, 51.0 mmol) in dry CH_2Cl_2 (80 mL) was added drop wise BBr_3 (30 mL, 1.0 M in CH_2Cl_2) at −78 °C. The reaction mixture was allowed to warm up to room temperature, poured into ice, and the aqueous portion was extracted with EtOAc and dried. It was further purified by column chromatography eluted with *n*-hexane:EtOAc (8:2) afforded **6** (8.25 g, 89%) as colorless solid, mp 67–69 °C; IR (neat) ν_{max} 2978, 1643, 1423, 1303, 1219, 1157, 1045, 937, 798 cm^{-1}; ^{1}HNMR (300 MHz, DMSO-d_6) δ 12.36 (1H, s, OH), 9.98 (1H, s, CHO), 6.11 (1H, d, J = 2.1 Hz), 6.06 (1H, d, J = 2.1 Hz), 3.84 (3H, s), 3.82 (3H, s); ^{13}CNMR (75 MHz, DMSO-d_6) δ 191.4, 168.1, 165.2, 163.4, 105.3, 93.2, 90.7, 56.1, 55.9; EIMS *m*/*z* (rel. int.): 182 (100) [M]$^+$, 181 (67), 164 (34), 151 (21), 136 (17), 69 (18); HREIMS calcd for $C_9H_{10}O_4$ [M]$^+$ 182.0579, found 182.0577.

5.2.2. 2-(Benzyloxy)-4,6-dimethoxybenzaldehyde (**7**)

A mixture of **6** (10.0 g, 55.0 mmol), 1-(chloromethyl)benzene (6.96 g, 55.0 mmol), and anhydrous K_2CO_3 (3.8 g, 27.5 mmol) in DMF (50 mL) were stirred at 80 °C for 6 h. The mixture was extracted with EtOAc and dried, and then concentrated to obtain suspension liquid. As hexane was added into this liquid, white solid precipitated and washed with water to obtain **7** (14.2 g, 95%), m.p. 91–93 °C; IR (neat) ν_{max} 2943, 2858, 1678, 1600, 1581, 1458, 1327, 945, 698, 648 cm^{-1}; ^{1}HNMR (300 MHz, $CDCl_3$) δ 10.38 (1H, s, CHO), 7.32 (5H, m), 6.08 (1H, s), 6.02 (1H, s), 5.08 (2H, s), 3.81 (3H, s), 3.77 (3H, s); ^{13}CNMR (75 MHz, $CDCl_3$) δ 187.1, 165.7, 163.2, 162.9, 135.7, 128.1, 127.5, 126.5, 108.6, 91.2, 90.2, 70.1, 55.5, 55.0; EIMS *m*/*z* (rel. int.): 272 [M]$^+$ (23), 243 (40), 181 (35), 92 (14), 91 (100), 61 (40); HREIMS calcd for $C_{16}H_{16}O_4$ [M]$^+$ 272.1049, found 272.1047.

5.2.3. 2-(Benzyloxy)-4,6-dimethoxyphenol (**8**)

To a solution of **7** (10.0 g, 36.8 mmol) in CH_2Cl_2 at room temperature, the *m*-CPBA (9.48 g, 55.1 mmol) was added slowly in portions. The reaction mixture was stirred for 4 h at room temperature and then the CH_2Cl_2 was removed under reduced pressure to obtain crude ester. This was hydrolyzed by NaOH (10%, 30 mL) in MeOH (30 mL) at room temperature stirring for 3 h. The reaction mixture was neutralized with 2 M HCl and extracted with EtOAc. The crude was further purified by column chromatography over a silica gel using *n*-hexane:EtOAc (8:2) to obtain brown red oil (5.2 g, 54%); IR (neat) ν_{max} 3460, 2943, 1697, 1600, 1512, 1458, 1319, 1149, 1103, 802 cm^{-1}; ^{1}HNMR (300 MHz, $CDCl_3$) δ 7.43–7.31 (5H, m), 6.21 (1H, s), 6.20 (1H, s), 5.09 (2H, s), 3.85 (3H, s), 3.71 (3H, s); ^{13}CNMR (75 MHz, $CDCl_3$) δ 152.8, 147.5, 146.2, 136.6, 129.5, 128.5, 128.1, 127.6, 93.4, 92.4, 71.4, 56.1, 55.6; EIMS *m*/*z* (rel. int.): 260 [M]$^+$ (14), 169 (100), 141 (39), 91 (66), 69 (12), 65 (14); HREIMS calcd for $C_{15}H_{16}O_4$ [M]$^+$ 260.1049, found 260.1052.

5.2.4. 1-(Benzyloxy)-2,3,5-trimethoxybenzene (**9**) from **8**

To a solution of **8** (10.0 g, 38.5 mmol), anhydrous K_2CO_3 (2.7 g, 19.5 mmol) and dimethyl sulfate (5.0 g, 39.6 mmol) in DMF (50 mL) was stirred at 80 °C for 6 h, cooled to room temperature, and then poured into H_2O (150 mL) and extracted with EtOAc. The crude product was further purified by silica gel column chromatography (*n*-hexane/EtOAc) to give **9** (6.2 g, 59%) as colorless oil; IR (neat) ν_{max} 2939, 2835, 1600, 1504, 1458, 1431, 1381, 1230, 1060, 948, 813, 744, 698 cm^1; ^{1}HNMR (300 MHz, $CDCl_3$) δ 7.38 (5H, m), 6.16 (2H, d, J = 3.0 Hz), 5.09 (2H, s), 3.80 (6H, s), 3.69 (3H, s); ^{13}CNMR (75 MHz, $CDCl_3$) δ 155.8, 153.6, 152.5, 136.9, 132.7, 128.2, 127.6, 127.0, 93.4, 92.1, 70.8, 60.7, 55.7, 55.1; EIMS *m*/*z* (rel. int.): 274 [M]$^+$ (59), 183 (22), 155 (98), 125 (19), 91 (100); HREIMS calcd for $C_{16}H_{18}O_4$ [M]$^+$ 274.1205, found 274.1208.

5.2.5. 3-Bromo-4-hydroxy-5-methoxybenzaldehyde (**13**)

To a solution of vanillin **12** (15.2 g, 100 mmol) in glacial acetic acid (75 mL) was added bromine (17.6 g, 110 mmol). After stirring for 1.5 h, the reaction mixture was diluted with ice water (200 mL). Precipitates were formed and filtered, washed with H_2O, and dried to give 5-bromvanillin **13** (21.5 g, 94%) as colorless solid, m.p. 162–164 °C; IR (neat) ν_{max} 3278, 1674, 1585, 1496, 1423, 1350, 1284, 1157, 1041, 968, 852, 786 cm^{-1}; ^{1}HNMR (300 MHz, DMSO-d_6) δ 10.72 (1H, s, OH), 9.75 (1H, s, CHO), 7.69 (1H, s), 7.39 (1H, s), 3.89 (3H, s); ^{13}CNMR (75 MHz, DMSO-d_6) δ 190.3, 149.7, 148.6, 128.9, 128.7, 109.5, 109.2, 56.3; EIMS *m*/*z* (rel. int.): 231 [M]$^+$ (82), 230 (100), 229 (74), 187 (12); HREIMS calcd for $C_8H_7O_3Br$ [M]$^+$ 229.9579, found 229.9581.

5.2.6. 3,4-Dihydroxy-5-methoxybenzaldehyde (**14**)

5-Bromovanilin **13** (20 g, 91 mmol), NaOH (24.5 g, 610 mol) and copper powder (0.1 g, 1.6 mmol) were slurred into water (300 mL). The reaction mixture was heated at reflux for 24–27 h. Disodium hydrogen phosphate (0.45 g, 3.2 mmol) was added at the last half hour of reflux. The reaction was then cooled at 50 °C, filtered to remove a precipitate of cupric hydrogen phosphate and acidified with HCl (46 g). The reaction mixture was extracted with EtOAc and separated by silica gel column chromatography using *n*-hexane:EtOAc (6:4) as eluents to yield **14** (9.5 g, 62%) as a colorless solid, m.p. 132–134 °C; IR (neat) ν_{max} 3278, 1674, 1593, 1523, 1462, 1334, 1207, 1141, 1091, 1002, 840, 717 cm^{-1}; ^{1}HNMR (300 MHz, DMSO-d_6) δ 9.68 (1H, s, CHO), 9.50 (1H, s, OH), 9.44 (1H, s, OH), 7.01 (2H, s), 3.81 (3H, s, OMe); ^{13}CNMR (75 MHz, DMSO-d_6) δ 191.3, 148.5, 146.0, 141.1, 127.4, 110.9, 105.0, 56.0; EIMS *m*/*z* (rel. int.): 168 [M]$^+$ (100), 167 (79), 125 (18), 97 (16); HREIMS calcd for $C_8H_8O_4$ [M]$^+$ 168.0423, found 168.0423.

*5.2.7. 3-Hydroxy-4,5-dimethoxybenzaldehyde (**15**)*

A mixture of **14** (25 g, 148.8 mmol), $(CH_3)_2SO_4$ (18.75 g, 148.8 mmol), and Na_2CO_3 (17.5 g, 165.1 mmol) was slurred into acetone and further reflux for 6 h. The resulting crude was further purified by column chromatography to obtain an oil and this was readily crystallized to give **15** (19.8 g, 73%), m.p. 65–67 °C; IR (neat) ν_{max} 3414, 2943, 2843, 1689, 1589, 1504, 1462, 1338, 1203, 1134, 995, 837, 752, 702 cm^{-1}; ^{1}HNMR (300 MHz, DMSO-d_6) δ 9.78 (1H, s, CHO), 9.71 (1H, s, OH), 7.03 (2H, s), 3.83 (3H, s, OMe), 3.76 (3H, s, OMe); ^{13}CNMR (75 MHz, DMSO-d_6) δ 191.8, 153.5, 151.0, 141.8, 131.6, 111.0, 104.3, 59.9, 55.8; EIMS *m/z* (rel. int.): 182 $[M]^+$ (100), 167 (35), 111 (26), 93 (11); HREIMS calcd for $C_9H_{10}O_4$ $[M]^+$ 182.0579, found 182.0576.

*5.2.8. 3-(Benzyloxy)-4,5-dimethoxybenzaldehyde (**16**)*

A mixture of **15** (10.0 g, 55 mmol), benzyl chloride (6.96 g, 55 mmol), and anhydrous K_2CO_3 (3.8 g, 27.5 mmol) in DMF (50 mL) was stirred at 80 °C for 6 h, and then poured into water and extracted with EtOAc and dried. It concentrated in *vacuo* to give **16** (13.8 g, 92%) as a pale yellow oil; IR (neat) ν_{max} 2939, 2831, 1689, 1585, 1496, 1458, 1384, 1323, 124, 995, 837, 732 cm^{-1}; ^{1}HNMR (300 MHz, $CDCl_3$) δ 9.63 (1H, s, CHO), 7.25 (2H, s), 7.17 (3H, t, $J = 7.2$ Hz), 6.98 (2H, m), 4.97 (2H, s), 3.77 (3H, s, OMe), 3.71 (3H, s, OMe); ^{13}CNMR (75 MHz, $CDCl_3$) δ 190.4, 153.2, 152.0, 143.5, 135.9, 131.1, 128.0, 127.5, 126.8, 108.3, 106.1, 70.4, 60.3, 55.5; EIMS *m/z* (rel. int.): 272 $[M]^+$ (37), 181 (15), 91 (100); HREIMS calcd for $C_9H_{10}O_4$ $[M]^+$ 272.1049, found 272.1050.

*5.2.9. 1-(Benzyloxy)-2,3,5-trimethoxybenzene (**9**) from **16***

To a solution of **16** (10.0 g, 36.8 mmol) in CH_2Cl_2 at room temperature, *m*-CPBA (9.48 g, 55.1 mmol) was added slowly in portions. The reaction mixture was stirred for 4 h at room temperature and then the CH_2Cl_2 was removed under reduced pressure to obtain ester crude product. It was hydrolyzed by aqueous 10% NaOH (30 mL) in MeOH (30 mL) at room temperature for 3 h. The reaction mixture was neutralized with 2 M HCl and extracted with EtOAc. The resulting crude without purification was directly used for further methylation with dimethyl sulfate in Me_2CO and anhydrous K_2CO_3 to obtain **9** in 30%.

*5.2.10. 4-(Benzyloxy)-2,3,6-trimethoxybenzaldehyde (**10**) and 2-(benzyloxy)-3,4,6- trimethoxybenzaldehyde (**11**)*

In a round-bottomed flask compound **9** (1.37 g, 5.0 mmol) was suspended in dry DMF (1.83 g, 25 mmol). The reaction flask was kept at ice-bath (0 °C). To this stirred reaction mixture, phosphorus oxychloride (3.06 g, 20 mmol) was added drop wise. The reaction mixture was further kept for 30 min in the cooling bath and then heated at 80 °C for 3 h. After completion, the reaction mixture was slowly poured into ice-cold water and then it was basified with 10% aqueous NaOH to precipitate. The regioisomeric mixture of aldehydes **10** (765 mg, 51%) and **11** (245 mg, 16%) were purified with the aid of silica gel column chromatography. **10**: m.p. 76–78 °C; IR (neat) ν_{max} 2939, 2862, 1678, 1593, 1462, 1396, 1334, 1249, 1199, 1041, 979, 910, 802, 748 cm^{-1}; ^{1}HNMR (300 MHz, $CDCl_3$) δ 10.30 (1H, s, CHO), 7.41 (5H, m), 6.30 (1H, s), 5.21 (2H, s), 3.96 (3H, s), 3.84 (3H, s), 3.81 (3H, s); ^{13}CNMR (75 MHz, $CDCl_3$) δ 188.0, 158.5, 158.2, 156.8, 136.2, 135.8, 128.7, 128.3, 127.2, 112.7, 93.3, 70.9, 62.1, 61.1, 56.0; EIMS *m/z* (rel. int.): 302 $[M]^+$ (20), 229 (18), 187 (22), 167 (21), 149 (45), 91 (100), 77 (15); HREIMS calcd for $C_{17}H_{18}O_5$ $[M]^+$ 302.1154, found 302.1155. **11**: m.p. 81–83 °C; IR (neat) ν_{max} 2939, 1681, 1593, 1462, 1369, 1334, 1249, 1207, 1138, 1041, 975, 902, 806, 748, 648 cm^{-1}; ^{1}HNMR (300 MHz, $CDCl_3$) δ 10.27 (1H, s, CHO), 7.47 (2H, d, $J = 9.0$ Hz), 7.35 (3H, m), 6.28 (1H, s), 5.14 (2H, s), 3.95 (3H, s), 3.89 (3H, s), 3.82 (3H, s); ^{13}CNMR (75 MHz, $CDCl_3$) δ 188.0, 159.1, 158.6, 155.4, 136.6, 136.0, 128.6, 128.4, 128.2, 112.9, 91.8, 76.5, 61.1, 56.1, 56.0; EIMS *m/z* (rel. int.): 302 $[M]^+$ (70), 273 (30), 259 (37), 210 (58), 197 (29), 195 (33), 181 (82), 153 (37), 91 (100), 65 (46); HREIMS calcd for $C_{17}H_{18}O_5$ $[M]^+$ 302.1154, found 302.1152.

*5.2.11. 1-(4-(Benzyloxy)-3-methoxyphenyl)ethanone (**19**)*

Compound **18** (8.3 g, 50 mmol) was dissolved in DMF (50 mL) and then anhydrous K_2CO_3 (2.5 g) and benzyl chloride (6.3 g, 50 mmol) were added. The reaction mixture was stirred at 80 °C for 6 h. The resulting crude was dissolved in H_2O and extracted with EtOAc and then was purified by silica gel column chromatography to obtain **19** (12 g, 94%) as a white powder, m.p. 82–84 °C; IR (neat) ν_{max} 2873, 1670, 1585, 1512, 1458, 115, 1350, 1276, 1215, 1145, 1076, 991, 871, 798, 748 cm^{-1}; ^{1}HNMR (300 MHz, $CDCl_3$) δ 7.57–7.33 (7H, m), 6.90 (1H, d, $J = 9.0$ Hz), 5.23 (2H, s), 3.95 (3H, s), 2.55 (3H, s); ^{13}CNMR (75 MHz, $CDCl_3$) δ 196.6, 152.2, 149.3, 136.1, 130.5, 128.5, 127.9, 127.0, 122.9, 111.9, 110.3, 70.6, 55.8, 26.0; EIMS *m/z* (rel. int.): 256 $[M]^+$ (39), 92 (26), 91 (100), 65 (24); HREIMS calcd for $C_{16}H_{16}O_3$ $[M]^+$ 256.1099, found 256.1096.

*5.2.12. (E)-3-(4-(Benzyloxy)-2,3,6-trimethoxyphenyl)-1-(4-(benzyloxy)-3- methoxyphenyl)- prop- 2-en-1-one (**20**)*

The chalcone **20** was prepared by base-catalyzed condensation of 1-(4-(benzyloxy)-3- methoxy phenyl)ethanone **19** (1.28 g, 5 mmol) with 4-(benzyloxy)-2,3,6-trimethoxy- benzaldehyde **10** (1.51 g, 5 mmol) in MeOH (30 mL). To a stirred reaction mixture at 0 °C was added a 30% aqueous solution of KOH (30 mL) drop wise over 30 min. The reaction mixture was kept at room temperature for 24 h, remove methanol under reduced pressure and extracted with EtOAc. The resulting crude was purified by column chromatography (benzene:acetone = 9:1) to obtain chalcone **20** as yellow oil (1.85 g, 69%); IR (neat) ν_{max} 2935, 1674, 1593, 1504, 1458, 1411, 1338, 1265, 1022, 802, 744 cm^{-1}; ^{1}HNMR (300 MHz, $CDCl_3$) δ 8.09 (1H, d, $J = 15.6$ Hz), 7.94 (1H, d, $J = 15.6$ Hz), 7.67 (1H, d, $J = 1.5$ Hz), 7.59 (1H dd, $J = 1.5$, 8.4 Hz), 7.41 (10H, m), 6.93 (1H, d, $J = 8.4$ Hz), 6.34 (1H, s), 5.26 (2H, s), 5.19 (2H, s), 3.98 (3H, s), 3.94 (3H, s), 3.87 (3H, s), 3.83 (3H, s).

*5.2.13. Viscolin (**1**)*

To a solution of chalcone **20** (1.50 g, 2.8 mmol) in a mixture of EtOAc:MeOH (9:1, 50 mL) was added 10% Pd–C (100 mg). This mixture was stirred at room temperature under H_2 gas atmosphere for 72 h, and then it was filtered. The liquid was concentrated and further purified by column chromatography over silica gel (Hexanes:EtOAc = 7:3) to give viscolin **1** (840 mg, 86%) as a colorless solid, m.p. 122–124 °C; IR (neat) ν_{max} 3425, 2935, 2839, 1600, 1512, 1462, 1423, 1269, 1195, 1149, 1091, 1033, 991 cm^{-1}; ^{1}HNMR (300 MHz, $CDCl_3$) δ 6.84 (1H, d, $J = 8.4$ Hz), 6.72 (1H, d, $J = 8.4$ Hz), 6.71 (1H, s), 6.32 (1H, s), 5.76 (1H, s, OH), 5.55 (1H, s, OH), 3.87 (3H, s), 3.85 (3H, s), 3.83 (3H, s), 3.75 (3H, s), 2.62 (4H, t, $J = 6.6$ Hz), 1.77 (2H, m); ^{13}CNMR (75 MHz, $CDCl_3$) δ 154.2, 151.2, 147.3, 146.2, 143.3, 134.8, 133.5, 120.8, 116.0, 114.0, 111.0, 94.3, 60.8, 60.5, 55.8, 55.6, 35.6, 31.9, 23.1; EIMS *m/z* (rel. int.): 348 $[M]^+$ (64), 198 (23), 197 (100), 137 (24); HREIMS calcd for $C_{19}H_{24}O_6$ $[M]^+$ 348.1573, found 348.1572.

5.3. Bioassay

5.3.1. Chemicals and antibodies

LPS (endotoxin from *Escherichia coli*, serotype 0127:B8), Carr (λ-carrageenin), Indo (indomethacin), MTT (3-[4,5-dimethylthiazol-2-yl]- 2,5-diphenyltetrazolium bromide) and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). TNF-α was purchased from Biosource International Inc. (Camarillo, CA, USA). Anti-iNOS, anti-COX-2, and anti-β-actin antibody (Santa Cruz, USA) and a protein assay kit (Bio-Rad Laboratories Ltd., Watford, Herts,

U.K.) were obtained as indicated. MAPK/extracellular signal-regulated kinase (ERK) 1/2, c-Jun NH_2-terminal kinase (JNK)/stress-activated protein kinase, and p38 MAPK proteins and phosphorylated proteins were purchased from Cell Signaling Technology (Beverly, MA). Poly-(vinylidene fluoride) membrane (Immobilon-P) was obtained from Millipore Corp. (Bedford, MA, USA).

5.3.2. Animals

Male imprinting control region (ICR) mice (6–8 weeks) were obtained from the BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan). The animals were kept in plexiglass cages at a constant temperature of 22 ± 1 °C, and relative humidity of 55 ± 5% with 12 h dark–light cycle for at least 2 week before the experiment. They were given food and water *ad libitum*. All experimental procedures were performed according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. After a 2 week adaptation period, male ICR mice (18–25 g) were randomly assigned to four groups ($n = 6$) of the animals in the study. The control group receives normal saline (i.p.). The other three groups include a Carr-treated, a positive control (Carr + Indo) and viscolin administered groups (Carr + viscolin).

5.3.3. Cell culture

A murine macrophage cell line RAW 264.7 (BCRC No. 60001) was purchased from the Bioresources Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in plastic dishes containing Dulbecco's Modified Eagle Medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) in a CO_2 incubator (5% CO_2 in air) at 37 °C and subcultured every 3 days at a dilution of 1:5 using 0.05% trypsin–0.02% EDTA in Ca^{2+}-, Mg^{2+}- free phosphate-buffered saline (DPBS).

5.3.4. Cell viability

Cells (2×10^5) were cultured in 96-well plate containing DMEM supplemented with 10% FBS for 1 day to become nearly confluent. Then cells were cultured with viscolin in the presence of 100 ng/mL LPS for 24 h. After that, the cells were washed twice with DPBS and incubated with 100 μL of 0.5 mg/mL MTT for 2 h at 37 °C testing for cell viability. The medium was then discarded and 100 μL dimethyl sulfoxide (DMSO) was added. After 30-min incubation, absorbance at 570 nm was read using a microplate reader.

5.3.5. Measurement of nitric oxide/nitrite

NO production was indirectly assessed by measuring the nitrite levels in the cultured media and serum determined by a colorimetric method based on the Griess reaction [23].

5.3.6. Carr-induced edema

The Carr-induced hind paw edema model was used for determination of anti-inflammatory activity according to the previous report [23].

5.3.7. Lipid peroxidation assay: malondialdehyde (MDA) formation

Determination of MDA from Carr-induced edema foot by the thiobarbituric acid reactive substances (TBARS) was used an index of the extent of lipid peroxidation [23].

5.3.8. Measurement of serum TNF-α and PGE_2 by an enzyme-linked Immunosorbent assay (ELISA)

Cell culture medium or serum levels of TNF-α and PGE_2 were determined using a commercially available ELISA kit (Biosource International Inc., Camarillo, CA) according to the manufacturer's instruction. The concentrations of TNF-α and PGE_2 were were expressed as pg/mL.

5.3.9. Antioxidant enzyme activity Measurements

Total SOD activity was determined by the inhibition of cytochrome *c* reduction [24]. Total CAT activity was based on that of Aebi [25]. Total GPx activity in cytosol was determined according to Paglia and Valentine's method [26].

5.3.10. Protein Lysate preparation and Western blot analysis

Total protein was extracted with a RIPA solution (radioimmunoprecipitation assay buffer) at −20 °C overnight. We used BSA (bovine serum albumin) as a protein standard to calculate equal total cellular protein amounts. Protein samples (30 μg) were resolved by denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using standard methods, and then were transferred to PVDF membranes by electroblotting and blocking with 1% BSA. The membranes were probed with the primary antibodies at 4 °C overnight, washed three times with PBST, and incubated for 1 h at 37 °C with horseradish peroxidase conjugated secondary antibodies. The membranes were washed three times and the immunoreactive proteins were detected by enhanced chemiluminescence (ECL) using hyperfilm and ECL reagent (Amersham International plc., Buckinghamshire, U.K.). The results of Western blot analysis were quantified by measuring the relative intensity compared to the control using Kodak Molecular Imaging Software and represented in the relative intensities.

5.3.11. Histological examination

For histological examination, biopsies of paws were taken 5 h following the intraplantar injection of Carr. The tissue slices were fixed in Dietric solution (14.25% ethanol, 1.85% formaldehyde, 1% acetic acid) for 1 week at room temperature, dehydrated by graded ethanol and embedded in Paraplast (Sherwood Medical). Sections (7 μm thick) were deparaffinized with xylene and stained with trichromic Van Gieson, and antigen retrieval was performed with citrate buffer, then blocked with 5% normal goat serum in PBS and incubated with rabbit anti-COX-2 and anti-*i*NOS in PBS with 5% normal goat serum. The sections were incubated with biotinylated goat anti-rabbit IgG. After washing in PBS, sections were processed with the Dako kit (Dako REALTM envision TM detection system). Thus some sections were stained with hematoxylin and eosin, while others were processed for *i*NOS and COX-2 immunohistochmistry staining. All samples were observed and photographed with BH2 Olympus microscopy. The numbers of neutrophils were counted in each scope (400 ×) and thereafter obtain their average count from five scopes of every tissue slice in hematoxylin and eosin stain.

5.3.12. Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical evaluation was carried out by one-way analysis of variance (ANOVA followed by Scheffe's multiple range tests). Statistical significance is expressed as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Acknowledgments

This work was supported by a grant of National Science Council, Taiwan, Republic of China, awarded to T. S. Wu. This study is supported in part by National Cheng Kung University (HUA100-3-2-232).

Appendix. Supplementary Data

Figs. S1–S6 were provided in the supporting information. Supplementary data related to this article can be found online at doi:10.1016/j.ejmech.2011.12.008.

References

[1] M.H. Pan, C.S. Lai, S. Dushenkov, C.T. Ho, J. Agric. Food Chem. 57 (2009) 4467–4477.
[2] K.J. Yun, D.J. Koh, S.H. Kim, S.J. Park, J.H. Ryu, D.G. Kim, J.Y. Lee, K.T. Lee, J. Agric. Food Chem. 56 (2008) 10265–10272.
[3] Y.L. Leu, T.L. Hwang, Y.M. Chung, P.Y. Hong, Chem. Pharm. Bull. 54 (2006) 1063–1066.
[4] S.T. Chiu, Flora of Taiwan, second ed.., vol. 2, Editorial Committee of the Flora of Taiwan, Taipei, 1996, 282–285.
[5] S.T. Chiu, Proceedings of International Symposium on Plant Biodiversity and development of Bioactive natural products, Taichung, Taiwan, Nov. 18-20, 2001, pp. 103–116.
[6] T.L. Hwang, Y.L. Leu, S.H. Kao, M.C. Tang, H.L. Chang, Free Radic. Biol. Med. 41 (2006) 1433–1441.
[7] D.R. Janero, Free Radic. Biol. Med. 9 (1990) 515–540.
[8] C.R. Su, Y.H. Shen, P.C. Kuo, Y.L. Leu, A.G. Damu, Y.H. Wang, T.S. Wu, Bioorg. Med. Chem. Lett. 16 (2006) 6155–6160.
[9] K. Lal, S. Ghosh, R.G. Salomon, J. Org. Chem. 52 (1987) 1072–1078.
[10] F. Roelens, K. Huvaere, W. Dhooge, M. van Cleemput, F. Comhaire, D. de Keukeleire, Eur. J. Med. Chem. 40 (2005) 1042–1051.
[11] T.J. Heckrodt, J. Mulzer, J. Am. Chem. Soc. 125 (2003) 4680–4681.
[12] D.V. Rao, F.A. Stuber, Synthesis 4 (1983) 308–310.
[13] P.S. Manchand, P.S. Belica, H.S. Wong, Synth. Commun. 20 (1990) 2659–2666.
[14] S. Hutchison, B.S. Choo-Kang, R.V. Bundick, A.J. Leishman, J.M. Brewer, I.B. McInnes, P. Garside, Clin. Exp. Immunol. 151 (2008) 114–122.
[15] C.J. Lee, L.G. Chen, W.L. Liang, C.C. Wang, Food Chem. 118 (2010) 315–322.
[16] H. Zhou, H. Lutterodt, Z. Cheng, L.L. Yu, J. Agric. Food Chem. 57 (2009) 4580–4585.
[17] L. Kole, B. Giri, S.K. Manna, B. Pal, S. Ghosh, Eur. J. Pharmacol. 653 (2011) 8–15.
[18] W.G. Spector, D.A. Willough, Bacteriol. Rev. 27 (1963) 117–154.
[19] C. Tohda, N. Nakayama, F. Hatanaka, K. Komatsu, Evidence-Based Compl. Altern. Med. 3 (2006) 255–260.
[20] C.S. Lai, J.H. Lee, C.T. Ho, C.B. Liu, J.M. Wang, Y.J. Wang, M.H. Pan, J. Agric. Food Chem. 57 (2009) 10990–10998.
[21] J. Dawson, A.D. Sedgwick, J.C. Edwards, P.A. Lee, Int. J. Tissue React. 13 (1991) 171–185.
[22] M.H. Huang, S.S. Huang, B.S. Wang, C.H. Wu, M.J. Sheu, W.C. Hou, S.S. Lin, G.J. Huang, J. Ethnopharmacol. 133 (2011) 743–750.
[23] H.Y. Chang, M.J. Sheu, C.H. Yang, Z.C. Leu, Y.S. Chang, W.H. Peng, S.S. Huang, G.J. Huang, Evidence-Based Compl. Altern. Med. (2011). doi:10.1093/ecam/nep027.
[24] L. Flohe, F. Otting, Meth. Enzymol. 105 (1984) 93–104.
[25] H. Aebi, Meth. Enzymol. 105 (1984) 121–126.
[26] E.D. Paglia, W.N. Valentine, J. Lab. Clin. Med. 70 (1967) 158–169.