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

Guan-Jhong Huang,[†,§] M. Vijaya Bhaskar Reddy,[‡,§] Ping-Chung Kuo,[⊥] Chieh-Hung Huang,[‡] Hung-Cheng Shih,[‡] E-Jian Lee,[Δ] Mei-Lin Yang,[‡] Yann-Lii Leu,[#] and Tian-Shung Wu*[,‡,∥]

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

---

* Author to whom correspondence should be addressed. Phone and fax, 886-6-2747538; e-mail: tswu@mail.ncku.edu.tw.

[†] School of Chinese Pharmaceutical Sciences, China Medical University.

[‡] National Cheng Kung University.

[⊥] National Formosa University.

[Δ] National Cheng Kung University Medical Center and Medical School.

[#] Chang Gung University.

[∥] Department of Pharmacy, China Medical University.

[§] Both authors provided equal contributions to this work.

Abbreviations: *i*NOS, inducible nitric oxide synthase; COX2, cyclooxygenase 2; LPS, lipopolysaccharide; NO, nitric oxide; TNF, tumor necrosis factor; PGE_2, prostaglandin E_2; MAPKs, mitogen-activated protein kinases; ERK, extracellular signal–regulated kinase; JNK, *c-Jun N*-terminal kinase; Carr, λ-carrageenin; ; Indomethacin, Indo; MDA, malondialdehyde; DMEM, Dulbecco's Modified Eagle's Medium; MTT, 3-(3,4-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; ELISA, enzyme-linked immunosorbent assay; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances; SOD, superoxide dismutase; CAT, catalase; SDS-PAGE, sodium doceyl sulfate polyacrylamide gel electrophoresis; cAMP, cyclic guanosine 3′,5′-monophosphate.

Abstract

Viscolin (**1**) is a 1, 3-diarylpropane purified from *Viscum coloratum* and reported to have affinity for the anti-inflammatory activity on superoxide anion generation ($O_2^{\cdot-}$) and elastase release in human neutrophils. In the present report, a concise synthesis of viscolin (**1**) has been achieved to construct the 1,3-diarylpropane skeleton employing selective demethylation and maintain the hydroxyl groups at *para* position in both rings, in which is the most important factor for the biological activity of viscolin (**1**).

In addition, anti-inflammatory effect of viscolin was investigated *in vitro* and *in vivo*. When RAW264.7 macrophages were treated with lipopolysaccharide (LPS) in the presence of viscolin, an inhibition of nitric oxide (NO), tumor necrosis factor-α (TNF-α) and PGE_2 productions were detected. Viscolin also blocked protein expression of inducible NO synthase (*i*NOS) and cyclooxygenase-2 (COX-2), and it also inhibited the extracellular signal–regulated kinase (ERK) for the activation of NF-κB in LPS-stimulated RAW264.7 macrophages. Viscolin also decreased the paw edema after λ-carrageenin (Carr) administration and increased the activities of antioxidant enzymes. We also demonstrated the viscolin attenuated the malondialdehyde (MDA) level in the edema paw and decreased the NO and TNF-α levels on the serum after Carr injection. Western blotting and immunohistochemical analysis revealed that viscolin decreased Carr-induced *i*NOS and COX-2 expressions. The anti-inflammatory mechanisms of viscolin might be related to the decrease in the level of MDA, *i*NOS, and COX-2 *via* increasing the activities of antioxidant enzymes in the edema paw through the suppression of TNF-α and NO.

Introduction

Inflammation leads to the signaling proteins in affected cells and tissues. Inducible nitric oxide synthase (*i*NOS), catalyzes the formation of nitric oxide (NO) from L-arginine.[1] Low concentration of NO produced by *i*NOS is likely to contribute to the antimicrobial activity of macrophages against certain bacterial pathogens. However, when overproduced in response to LPS stimulation, excess NO can react with superoxide anion radicals to form peroxynitrite, which is a stronger oxidant than NO and cause a high oxidative stress state. It 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 PGE_2 production stands as an important therapeutic goal.

Viscolin **1** is a naturally occurring 1, 3-diarylpropane and it consists of highly substituted aromatic ring with hydroxyl group at C-4 position in ring-A and 4-hydroxy-3-methoxy groups in ring-B joined through a propane linkage. It was identified from *Viscum coloratum* (Loranthaceae),[3] an 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 ($O_2^{\cdot-}$) 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] These effects are attributed to an elevation of cellular cAMP (cyclic guanosine 3′,5′-monophosphate) through inhibition of the activity of cAMP. A similar mechanism of inhibition may be used for the control of neutrophil-mediated tissue injury. 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]

It has been reported that inflammatory effect induced by Carr could be associated with free radicals in the previous literature. Free radical will be released when administrating with Carr for 1~5 hr. The edema effect was raised to maximum at the 3th hr and its MDA production was due to free radical attack plasma membrane.[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] The method described herein provides a shorter and higher yields route for the synthesis of viscolin and therefore it is beneficial for the further studies of the bioactivity of viscolin. With the assistance of this concise synthetic method, the anti-inflammatory effects of viscolin on LPS-induced in RAW264.7 cells and Carr-induced on paw edema in mice could be examined. The levels of iNOS and COX-2 in either RAW264.7 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.

Chemistry

The retrosynthetic analysis of viscolin **1** was illustrated in Figure 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 Figure 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-demethylated 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 sulphate 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 sulphate 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-trimethoxybenzaldehyde, 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 sulphate to yield **9** in 30 %. Both the routes for the preparation of substrate **9** provided the desired products in good yields ranging from 12−27 %. 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**. 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]

Results and Discussion

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 hr 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. 4A). 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 hr, a significant concentration-dependent inhibition of nitrite production was detected (Fig. 4B). 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 hr, the TNF-α concentration increased in the medium. When RAW264.7 macrophages were treated with different concentrations of viscolin (0, 5, 10, and 20 μM) together with LPS (100 ng/mL) for 24 hr, a significant concentration-dependent inhibition of TNF-α level was detected (Fig. 4C). 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. 4D). The IC_{50} value for inhibition of PGE_2 level of viscolin was about 14.37 ± 0.12 μM.

Effects of viscolin on the LPS-stimulated activation of *i*NOS, 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, RAW264.7 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 pro-inflammatory enzymes and to the production of pro-inflammatory 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 hr inhibit *i*NOS and COX-2 proteins expression in mouse macrophage RAW264.7 cells in a dose-dependent manner (Fig. 5A). 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

209 average of 83.8 and 70.6 % down-regulation of *i*NOS and COX-2 proteins, respectively, after
210 treatment with viscolin at 20 μM compared with the LPS-alone (Fig. 5B). These *in vitro* data
211 showed that viscolin suppressed LPS-induced productions of NO, TNF-α, and PGE_2, which
212 are the expression products of inflammatory protein such as *i*NOS and COX-2.
213
214 MAPKs play critical roles in the regulation of cell growth and differentiation, and control
215 cellular responses to cytokines and stresses. In particular, ERK, p38, and JNK are known to
216 be important for the activation of NF-κB.[17] To explore whether the inhibition of NF-κB
217 activation by viscolin is mediated through the MAPK pathway, MAPK phosphorylation was
218 examined by Western blot in RAW 264.7 cells pretreated with viscolin and then with LPS. As
219 shown in Fig. 6, we showed that ERK, JNK, and p38 were phosphorylated with LPS
220 stimulation. Furthermore, phosphorylation of ERK was inhibited by viscolin at 30min of LPS
221 stimulation, whereas there was no effect of viscolin on p-p38 or p-JNK. MAPKs are also
222 likely targets for the development of novel anti-inflammatory drugs; however, signaling from
223 MAPKs to transcription factors mediating iNOS and COX-2 expression is not fully
224 understood.
225
226 **Effects of viscolin on Carr-induced mice paw edema.** Since viscolin effectively
227 inhibited iNOS and COX-2 expressions in macrophages, studies were extended to determine
228 whether viscolin affected acute phase inflammation in animal models. In the present study, the
229 Carr-induced edema model was performed due to its widely adaptation for screening the
230 effects of anti-inflammatory drugs. Carr-induced paw edema is shown in Fig. 7A. Viscolin (8

mg/kg) significantly inhibited ($p < 0.001$) the development of paw edema induced by Carr after 4^{th} and 5^{th} hr of treatment, comparable with the reference compound Indo (10 mg/kg). Similarly, the MDA level increased significantly in the edema paw at 5^{th} 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. 7B). In addition, the NO and TNF-α levels increased significantly in the edema serum at 5^{th} h 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 5^{th} hr after induction (Fig. 7C and 7D).

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 3^{th} 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

256 further release of kinins and leukotrienes, which is suggested to be an important role in the
257 maintenance of long-lasting nociceptive response.[21] In the above results, viscolin significantly
258 decreased the TNF-α level in serum after Carr injection by treatment with 2, 4, and 8 mg/kg,
259 respectively. It suggested that anti-inflammatory effects of viscolin were resulted from the
260 inhibition of the proinflammatory cytokines in the Carr-induced paw edema.
261
262 **Effects of viscolin on activities of antioxidant enzymes.** The acute inflammatory
263 response is associated with the production of reactive oxygen species (ROS), which have been
264 proposed to mediate cell damage in the paw tissue. At 5th hr after the intrapaw injection of
265 Carr, paw tissues were analyzed for the biochemical parameters, such as CAT, SOD, and GPx
266 activities. CAT, SOD, and GPx activities in paw tissue were decreased significantly by Carr
267 administration. CAT, SOD, and GPx activity were increased significantly after treated with 8
268 mg/kg viscolin and 10 mg/kg Indo ($p < 0.01$) (Table 1). This local acute inflammation model
269 induces a biphasic edema consisting of an early phase (up to 2 hr) followed by a more
270 sustained late phase (2–6 hr). The early phase of Carr edema is related to the production of
271 immediate inflammation mediators such as histamine, bradykinin, leukotrienes,
272 platelet-activating factor and cyclooxygenase products in the inflamed tissue. The late phase
273 is related to neutrophil infiltration and the production of ROS. In a number of
274 pathophysiological conditions associated with inflammation or oxidant stress, these ROS have
275 been proposed to mediate cell damage via a number of independent mechanisms including the
276 initiation of lipid peroxidation, the inactivation of a variety of antioxidant enzymes and
277 depletion of glutathione. Giving the importance of the oxidative status in the formation of
278 edema, the anti-inflammatory effect exhibited by drug in this model might be related to its
279 antioxidant properties.[22] In our study, there is a significantly increment in CAT, SOD, and
280 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.

Effects of viscolin on Carr-induced *i*NOS 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 5^{th} hr inhibited *i*NOS and COX-2 proteins expression in mouse paw edema (Fig. 8A). 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. 8B). 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 iNOS and COX-2 activity of viscolin (8 mg/kg) exhibited as well as Indo (10.0 mg/kg) did.

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. 9A). Neutrophils were increased with Carr treatment (Fig. 9B). Indo and

viscolin (8 mg/kg) could decrease the neutrophils numbers as compared to the Carr-treated group (Fig. 9C and 9D). At 5^{th} h after intraplantar Carr injection, numerous iNOS and COX-2 immunoreactive cells were observed in the brown site of paw tissue (Fig. 9F and 9J). Administration of Indo and viscolin (8 mg/kg) 30 min prior to the Carr injection markedly reduced the increase in *i*NOS amd COX-2 immunoreactive cells in paws (Fig. 9G, 9H, 9K, and 9L).

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 RAW264.7 macrophages and a mouse model of topical inflammation respectively. The anti-inflammatory mechanism of viscolin may be related to iNOS, 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.

Experimental Section

General. 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}C NMR 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.

Synthesis of 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 dropwise BBr_3 (30 mL, 1.0 M in CH_2Cl_2) at -78^{o}C. The reaction mixture was allowed to warm up to room temperature for 2h, and poured into ice. The organic solvent was removed under reduced pressure and the aqueous portion was extracted with EtOAc. The combined organic phases were washed with brine and dried over anhydrous $MgSO_4$ and further purified by column chromatography eluted with *n*-hexane:EtOAc (8:2) afforded **6** (8.25 g, 89 %) as colorless solid, m.p. 67-69 ℃; 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}C NMR (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.

Synthesis of 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 ^{o}C for 6 h. The inorganic salts were removed by

filtration and extracted with EtOAc, dried over anhydrous Na_2SO_4 and concentrated in *vacuo* 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 ℃; 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}C NMR (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.

Synthesis of 2-(benzyloxy)-4,6-dimethoxyphenol (8). To a solution of **7** (10.0 g, 36.8 mmol) in CH_2Cl_2 at room temperature, and 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 2M 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}C NMR (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.

Synthesis of 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 dimethylsulphate (5.0 g, 39.6

mmol) in DMF (50 mL) was stirred at 80^{O}C for 6 h, cooled to room temperature, and then poured into H_2O (150 mL) and extracted with EtOAc. Washed the organic solvents with brines, dried over anhydrous Na_2SO_4 and concentrated in *vacuo* gave crude product. This 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}C NMR (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.

Synthesis of 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 ℃; 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.

Synthesis of 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 ℃, 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 ℃; 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

Synthesis of 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 inorganic salts were removed by filtration and evaporation acetone solvent. 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 ℃; 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.

Synthesis of 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 ℃ for 6 h, and then poured into water and extracted with

EtOAc, dried over anhydrous Na_2SO_4, and concentrated in *vacu*o to give **1**6 (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.

Synthesis of 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 2M HCl and extracted with EtOAc. The resulting crude without purification was directly used for further methylation with dimethylsulphate in Me_2CO and anhydrous K_2CO_3 to obtain **9** in 30 %.

Synthesis of 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 ℃). 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 ℃ 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 ℃; 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 ℃; 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.

Synthesis of 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 ℃ for 6 h. The inorganic salts were removed by filtration, and the resulting crude was dissolved in H_2O and extracted with EtOAc. The extract was purified by silica gel column chromatography to obtain **19** (12 g, 94%) as a white powder, m.p. 82-84 ℃; 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.

Synthesis of (*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-methoxyphenyl)ethanone **19** (1.28 g, 5 mmol) with 4-(benzyloxy)-2,3,6-trimethoxybenzaldehyde **10** (1.51 g, 5 mmol) in MeOH (30 mL). To a stirred reaction mixture at 0 ℃ was added a 30 % aqueous solution of KOH (30 mL) dropwise 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).

Synthesis of 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 under reduced pressure and the residue was 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 ℃; 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.

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).

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℃, 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).

Cell culture. A murine macrophage cell line RAW264.7 (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℃ 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).

Cell viability. Cells (2 x 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℃ 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.

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] The cells were incubated with viscolin (0, 5, 10 and 20 μM) in the presence of LPS (100 ng/mL) at 37 ℃ for 24 h. Then, cells were dispensed into 96-well plates, and 100 μL of each supernatant was mixed with the same volume of Griess reagent and incubated at room temperature for 10 min, the absorbance was measured at 540 nm with a Micro-Reader (Molecular Devices, Orleans Drive, Sunnyvale, CA). Serum samples were diluted four times with distilled water and deproteinized by adding 1/20 volume of zinc sulfate (300 g/L) to a final concentration of 15 g/L. After centrifugation at 10,000×*g* for 5 min at room temperature, 100 μL supernatant was applied to a microtiter plate well, followed by 100 μL of Griess reagent. After 10 min of color development at room temperature, the

absorbance was measured at 540 nm with a Micro-Reader. By using sodium nitrite to generate a standard curve, the concentration of nitrite was measured by absorbance at 540 nm.

Carr-induced Edema. The Carr-induced hind paw edema model was used for determination of anti-inflammatory activity.[23] Animals were i.p. treated with viscolin (5, 10, and 20 μM, equivalent to 2, 4, and 8 mg/kg), Indo or normal saline, 30 min prior to injection of 1% Carr (50 μL) in the plantar side of right hind paws of the mice. The paw volume was measured immediately after Carr injection and at 1, 2, 3, 4 and 5 hr intervals after the administration of the edematogenic agent using a plethysmometer (model 7159, Ugo Basile, Varese, Italy). The degree of swelling induced was evaluated by the ratio a/b, where a was the volume of the right hind paw after Carr treatment, and b was the volume of the right hind paw before Carr treatment. Indo was used as a positive control. After 5^{th} hr, the animals were sacrificed and the Carr-induced edema feet were dissected and stored at -80 ℃. Also, blood were withdrawn and kept at -80 ℃. The protein concentration of the sample was determined by the Bradford dye-binding assay (Bio-Rad, Hercules, CA).

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] The amount of MDA formed during the incubation was assessed by adding 1.5 % thiobarbituric acid and then heating at 95 ℃ for 45 min. After cooling, the samples were centrifuged, and the absorbance of TBARS in the supernatant was measured at 532 nm. The levels of lipid peroxidation are expressed in terms of TBARS nmol/mg protein.

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. TNF-α and PGE_2 were determined from a standard curve. The concentrations were expressed as pg/mL.

Antioxidant Enzyme Activity Measurements. Total SOD activity was determined by the inhibition of cytochrome *c* reduction.[24] The reduction of cytochrome *c* was mediated by superoxide anions generated by the xanthine/xanthine oxidase system and monitored at 550 nm. One unit of SOD was defined as the amount of enzyme required to inhibit the rate of cytochrome *c* reduction by 50%. Total CAT activity was based on that of Aebi.[25] In brief, the reduction of 10mM H_2O_2 in 20 mM of phosphate buffer (pH 7.0) was monitored by measuring the absorbance at 240 nm. The activity was calculated using a molar absorption coefficient, and the enzyme activity was defined as nanomoles of dissipating hydrogen peroxide per milligram protein per minute. Total GPx activity in cytosol was determined according to Paglia and Valentine's method.[26] The enzyme solution was added to a mixture containing hydrogen peroxide and glutathione in 0.1 mM Tris buffer (pH 7.2) and the absorbance at 340 nm was measured. Activity was evaluated from a calibration curve, and the enzyme activity was defined as nanomoles of NADPH oxidized per milligram protein per minute.

Protein Lysate Preparation and Western blot Analysis. Total protein was extracted with a RIPA solution (radioimmuno-precipitation assay buffer) at -20 ℃ 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 ℃ overnight, washed three times with PBST, and incubated for 1 hr at 37 ℃ 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.

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-iNOS 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 iNOS and COX-2 immunohistochmistry staining. All samples were observed and photographed with BH2 Olympus microscopy. Every three to five tissue slices were randomly chosen from Control, Carr-, Indo- and viscolin-treated (8 mg/kg) groups. 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.

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 $^{***}p < 0.001$.

Acknowledgements. This work was supported by a grant of National Science Council, Taiwan, Republic of China, and Grant No. (OUA 95-3-2-021) from the National Cheng Kung University, Tainan, Taiwan R.O.C awarded to T. S. Wu. This study is supported in part by Taiwan Department of Health Clinical Trial and Research Center of Excellence (DOH100-TD-B-111-004). Authors are also grateful to the National Center for High-performance Computing, Taiwan, Republic of China, for computer time and facilities.

References

1. Pan, M. H.; Lai, C. S.; Dushenkov, S.; Ho, C. T. Modulation of inflammatory genes by natural dietary bioactive compounds. *J. Agric. Food Chem.* **2009**, *57*, 4467–4477.
2. Yun, K. J.; Koh, D. J.; Kim, S. H.; Park, S. J.; Ryu, J. H.; Kim, D. G.; Lee, J. Y.; Lee, K. T. Anti-inflammatory effects of sinapic acid through the suppression of inducible nitric oxide synthase, cyclooxygase-2, and proinflammatory cytokines expressions via nuclear factor-κB inactivation. *J. Agric. Food Chem.* **2008**, *56*, 10265–10272.
3. Lew, Y. L.; Hwang, T. L.; Chung, Y. M.; Hong, P. Y. The inhibition of superoxide anion generation in human neutrophils by *Viscum coloratum*. *Chem. Pharm. Bull.* **2006**, *54*, 1063–1066.
4. Chiu, S. T. In *Flora of Taiwan*, 2nd ed., Vol. 2, Editorial Committee of the Flora of Taiwan, Taipei, pp. 282–285, 1996.
5. Chiu, S. T. Medicinal resources of formosan mistletoes, In *Proceedings of International*

Symposium on Plant Biodiversity and Development of Bioactive Natural Products, Taichung, Taiwan, Nov. 18-20, 2001, pp. 103–116.

6. Hwang, T. L.; Leu, Y. L.; Kao, S. H.; Tang, M. C.; Chang, H. L. Viscolin, a new chalcone from *Viscum coloratum*, inhibits human neutrophil superoxide anion and elastase release via a cAMP-dependent pathway. *Free Radical Biol. Med.* **2006**, *41*, 1433–1441.

7. Janero, D. R. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Fee Radical Bio. Med.* **1990**, *9*, 515–540.

8. Su, C. R.; Shen, Y. H.; Kuo, P. C.; Leu, Y. L.; Damu, A. G.; Wang, Y. H.; Wu, T. S. Total synthesis and biological evaluation of viscolin, a 1,3-diphenylpropane as a novel potent anti-inflammatory agent. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 6155–6160.

9. Lal, K.; Ghosh, S.; R. G. Salomon. Hydroxyl-directed regioselective monodemethylation of polymethoxyarenes. *J. Org. Chem.* **1987**, *52*, 1072–1078.

10. Roelens, F.; Huvaere, K.; Dhooge, W.; Van Cleemput, M.; Comhaire, F.; De Keukeleire, D. Regioselective synthesis and estrogenicity of (+/-)-8-alkyl-5,7-dihydroxy-4-(4-hydroxyphenyl)-3,4-dihydrocoumarins. *Eur. J. Med. Chem.* **2005**, *40*, 1042–1051.

11. Heckrodt, T. J.; Mulzer, J. Total synthesis of elisabethin A: intramolecular Diels−Alder reaction under biomimetic conditions. *J. Am. Chem. Soc.* **2003**, *125*, 4680–4681.

12. Rao, D. V.; Stuber, F. A. An efficient synthesis of 3,4,5-trimethoxybenzaldehyde from vanillin. *Synthesis* **1983**, *4*, 308–310.

13. Manchand, P. S.; Belica, P. S.; Wong, H. S. Synthesis of 3,4,5-trimethoxybenzaldehyde. *Synth. Commun.* **1990**, *20*, 2659–2666.

14. Hutchison, S.; Choo-Kang, B. S.; Bundick, R. V.; Leishman, A. J.; Brewer, J. M.; McInnes, I. B.; Garside, P. Tumour necrosis factor-alpha blockade suppresses murine

allergic airways inflammation. *Clin. Exp. Immunol.* **2008**, *151*, 114–122.

15. Lee, C. J.; Chen, L. G.; Liang, W. L.; Wang, C. C. Anti-inflammatory effects of *Punica granatum* Linne *in vitro* and *in vivo*. *Food Chem.* **2010**, *118*, 315–322.

16. Zhou, H.; Lutterodt, H.; Cheng, Z.; Yu, L. L. Anti-inflammatory and antiproliferative activities of trifolirhizin, a flavonoid from *Sophora flavescens* roots. *J. Agric. Food Chem.* **2009**, *57*, 4580–4585.

17. (ref. 35)

18. Spector, W. G.; Willoughb, D. A. The inflammatory response. *Bacteriol Rev.* **1963**, *27*, 117–154.

19. Tohda, C.; Nakayama, N.; Hatanaka, F.; Komatsu, K. Comparison of anti-inflammatory activities of six *Curcuma* rhizomes: a possible curcuminoid-independent pathway mediated by *Curcuma phaeocaulis* extract. *Evidence-Based Compl. Altern. Med.* **2006**, *3*, 255–260.

20. Lai, C. S.; Lee, J. H.; Ho, C. T.; Liu, C. B.; Wang, J. M.; Wang, Y. J.; Pan, M. H. Rosmanol potently inhibits lipopolysaccharide-induced *i*NOS and COX-2 expression through downregulating MAPK, NF-κB, STAT3 and C/EBP signaling pathways. *J. Agric. Food Chem.*, **2009**, *57*, 10990–10998.

21. Dawson, J.; Sedgwick, A. D.; Edwards, J. C.; Lees, P. A comparative study of the cellular, exudative and histological responses to carrageenan, dextran and zymosan in the mouse. *Int. J. Tissue React.* **1991**, *13*, 171–185.

22. Huang, M. H.; Huang, S. S.; Wang, B. S.; Wu, C. H.; Sheu, M. J.; Hou, W. C. Lin, S. S. Huang, G. J. Antioxidant and anti-inflammatory properties of *Cardiospermum halicacabum* and its reference compounds *ex vivo* and *in vivo*. *J. Ethnopharmacol.* **2011**, *133*, 743–750.

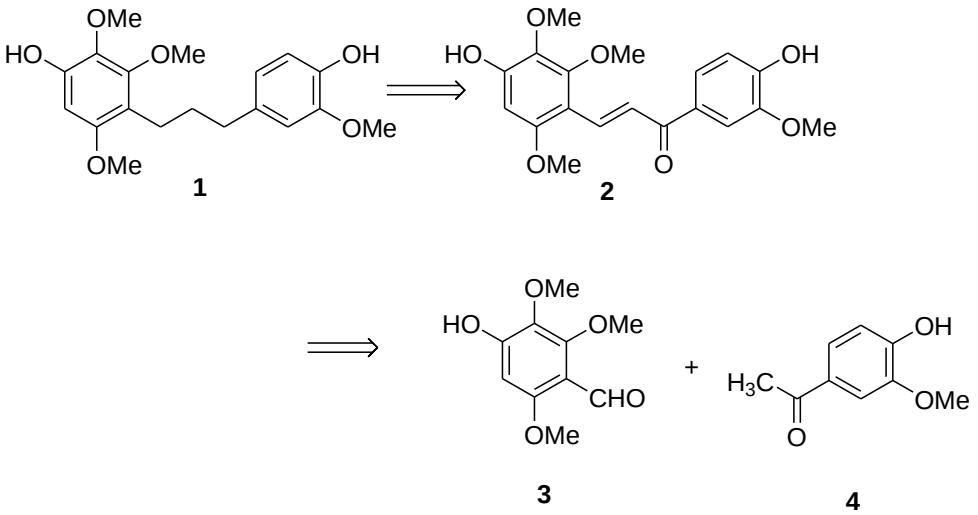
23. Chang, H. Y.; Sheu, M. J.; Yang, C. H.; Leu, Z. C.; Chang, Y. S.; Peng, W. H.; Huang, S.

S.; Huang, G. J. Analgesic effects and the mechanisms of anti-inflammation of hispolon in mice. *Evidence-Based Compl. Altern. Med.* **2009**, doi:10.1093/ecam/nep027.

24. Flohe, L.; Otting, F. Superoxide dismutase assays. *Meth. Enzymol.* **1984**, *105*, 93–104.

25. Aebi, H. Catalase *in vitro*. *Meth. Enzymol.* **1984**, *105*, 121–126.

26. Paglia, E. D.; Valentine, W. N. Studies on the quantitative and qualitative characterization of erythrocytes glutathione peroxidase. *J. Lab. Clin. Med.* **1967**, *70,* 158–169.

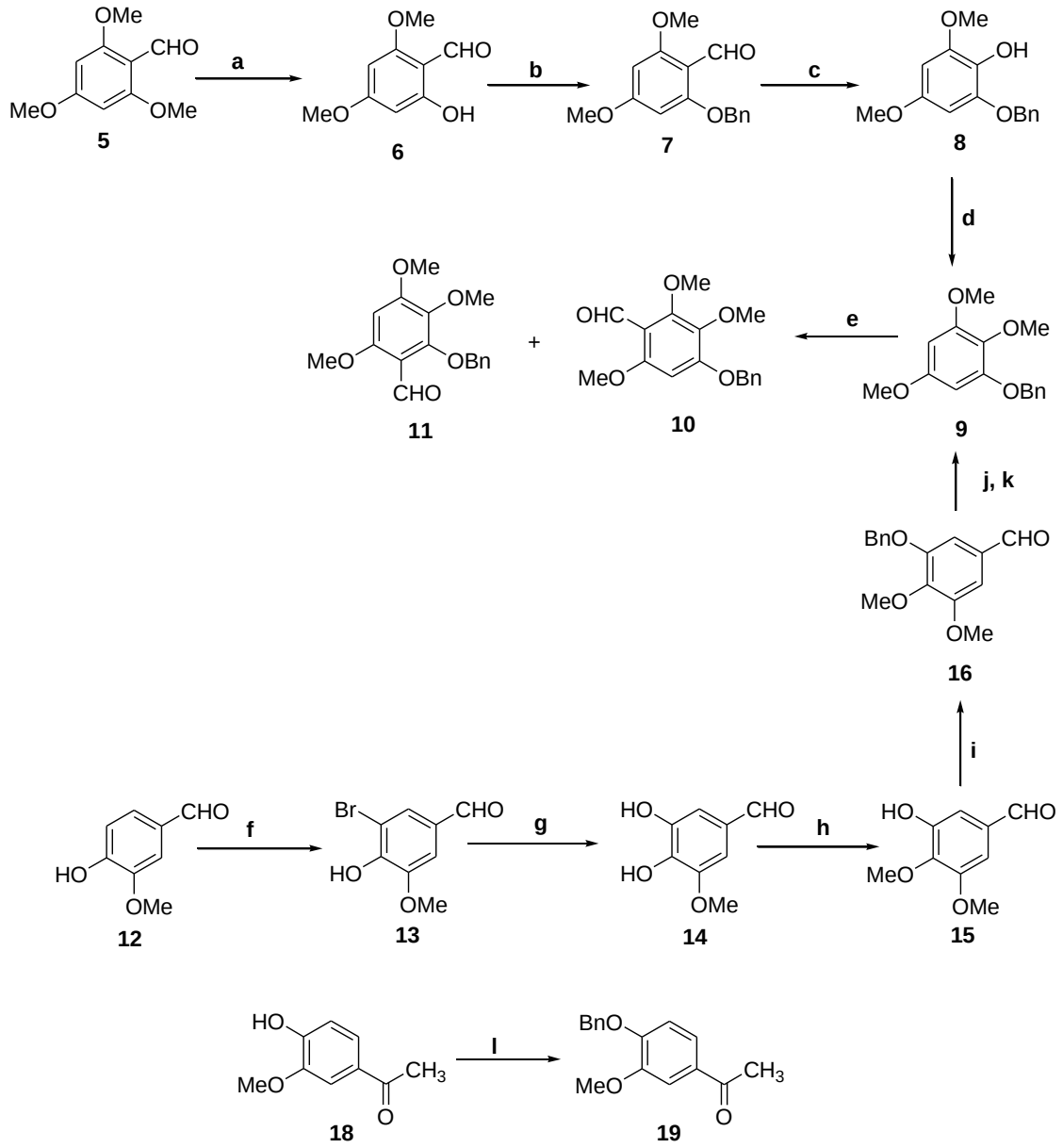


712

713 **Figure 1.** Retrosynthetic analysis of viscolin **1**.

714

715

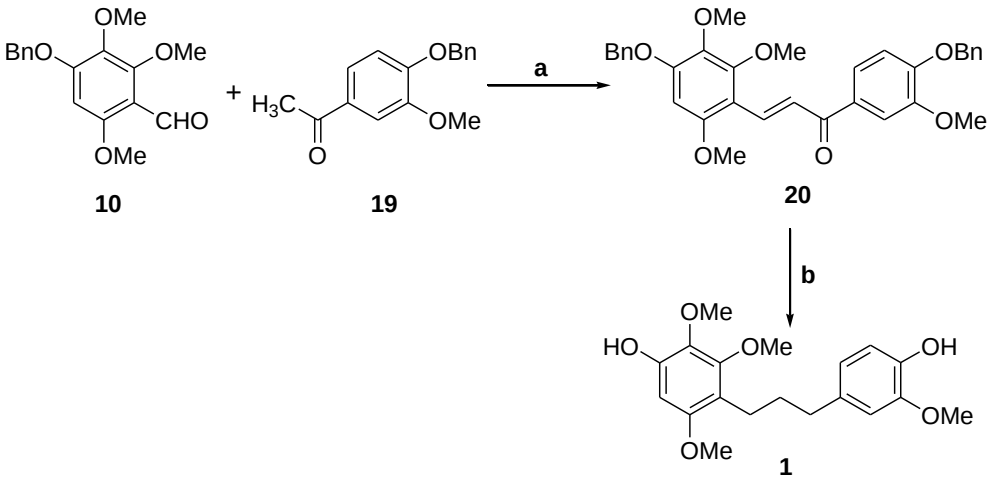


Reagents and conditions: **a**) BBr_3 in 1.0 M CH_2Cl_2, -78^{0}C, 1.5 h; **b**) BnCl, DMF, K_2CO_3, reflux, 6h; **c**) (1) *m*-CPBA, CH_2Cl_2, room temp, 4 h; (2) NaOH-MeOH, room temp, 3 h; **d**) $(CH_3)_2SO_4$, K_2CO_3, reflux, 4h; **e**) $POCl_3$, DMF; **f**) Br_2/acetic acid, room temp.; **g**) NaOH solution in Cu power, reflux; **h**) dimethyl sulphate, Na_2CO_3, reflux; **i**) BnCl, DMF, K_2CO_3, reflux, 6 h; **j**) (1) *m*-CPBA, CH_2Cl_2, room temp, 4 h, (2) NaOH-MeOH, room temp, 3 h; **k**) $(CH_3)_2SO_4$, K_2CO_3, reflux, 4 h; **l**) BnCl, DMF, K_2CO_3, reflux, 6 h.

716
717

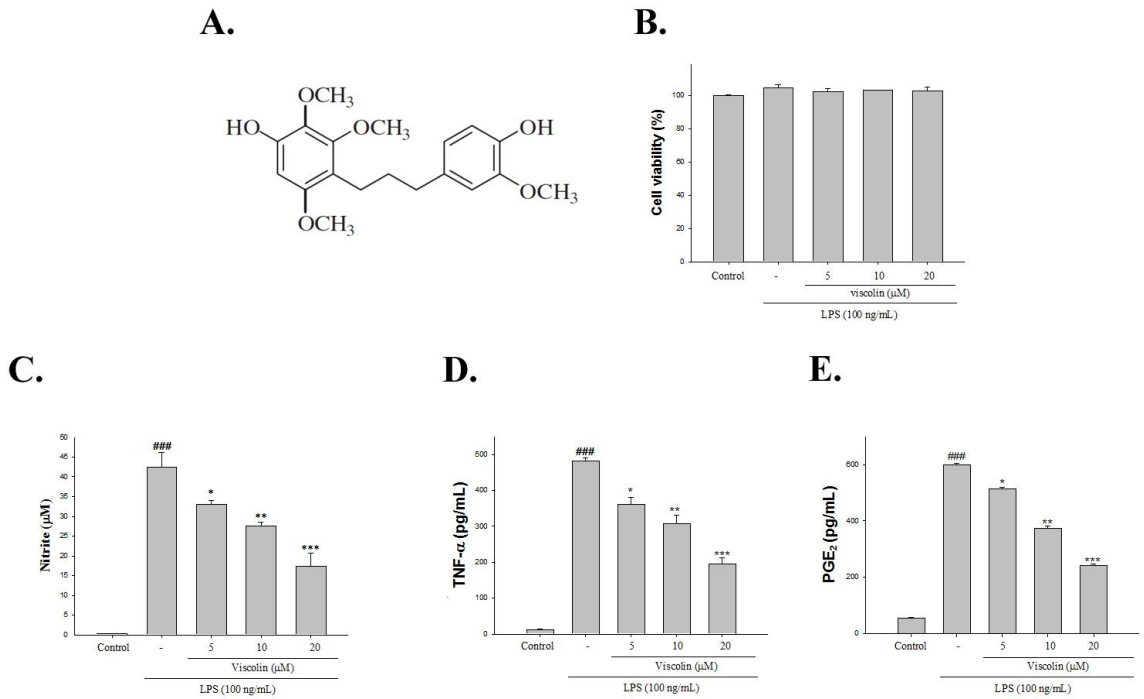
718 **Figure 2.** Preparation of the precursors **9**, **10**, and **19**.

719



Reagents and conditions: **a**) 30% KOH, methanol, room temp, 48 h; **b**) 10% Pd/C, H_2, EtOAc:MeOH (9:1), 72 h.

Figure 3. Synthesis of viscolin **1**.



723

724

725 **Figure 4.** (A) cytotoxic effects of viscolin in RAW264.7 cells and effects of viscolin on

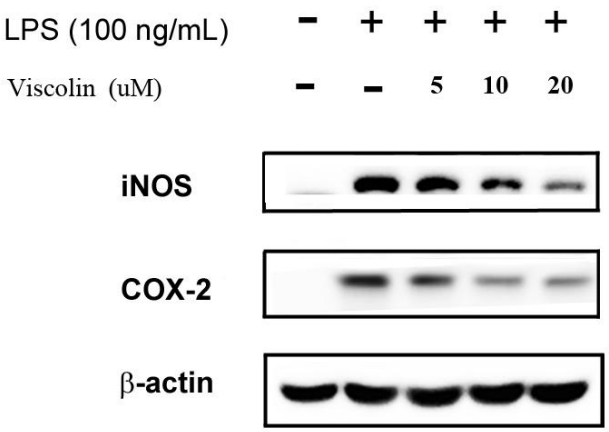
726 LPS-induced NO (B), TNF-α (C), and PGE_2 (D) productions of RAW264.7 macrophages.

727 The data were presented as mean ± S.D. for three different experiments performed in

728 triplicate. ### compared with sample of control group. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$

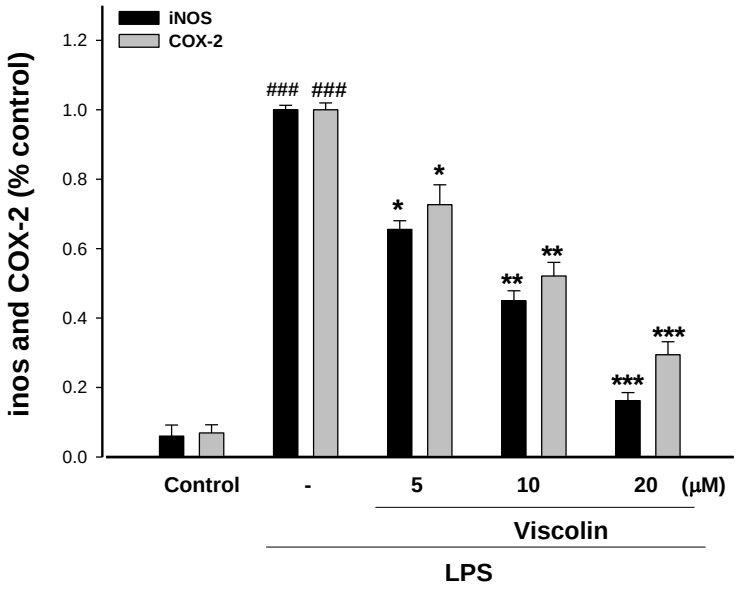
729 were compared with LPS-alone group.

730



731

732 (A)



733

734 (B)

735 **Figure 5.** Inhibition of iNOS and COX-2 protein expressions by viscolin in LPS-stimulated
736 RAW264.7 cells. (A) A representative Western blot from two separate experiments is shown.
737 (B) Relative *i*NOS and COX-2 protein levels were calculated with reference to a
738 LPS-stimulated culture. $^{###}$ Compared with sample of control group. The data were presented
739 as mean ± S.D. for three different experiments performed in triplicate. $^{*}p < 0.05$ and $^{***}p <$
740 0.001 were compared with LPS-alone group.

741

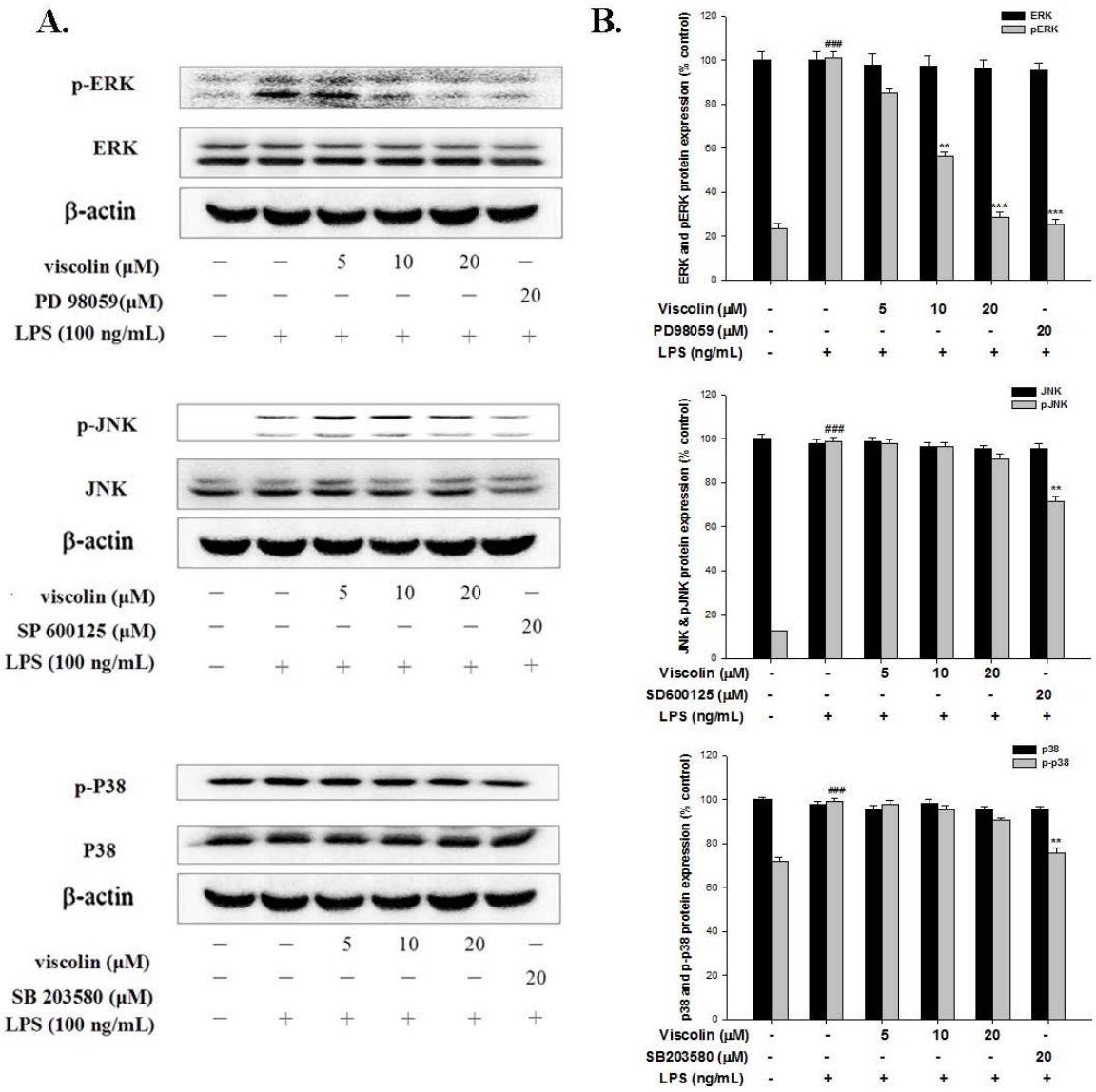


Figure 6. Inhibition of Phosphorylation of MAPKs protein expressions by viscolin in LPS-stimulated RAW264.7 cells. Lysed cells were then prepared and subjected to Western blotting using an antibody specific for p-ERK1/2, ERK1/2, p-p38 MAPK, p38 MAPK, p-JNK and JNK. β-actin was used as an internal control. (A) A representative Western blot from two separate experiments is shown. (B) Relative MAPKs protein levels were calculated with reference to a LPS-stimulated culture. ### Compared with sample of control group. The data were presented as mean ± S.D. for three different experiments performed in triplicate. $^{*}p < 0.05$ and $^{***}p < 0.001$ were compared with LPS-alone group.

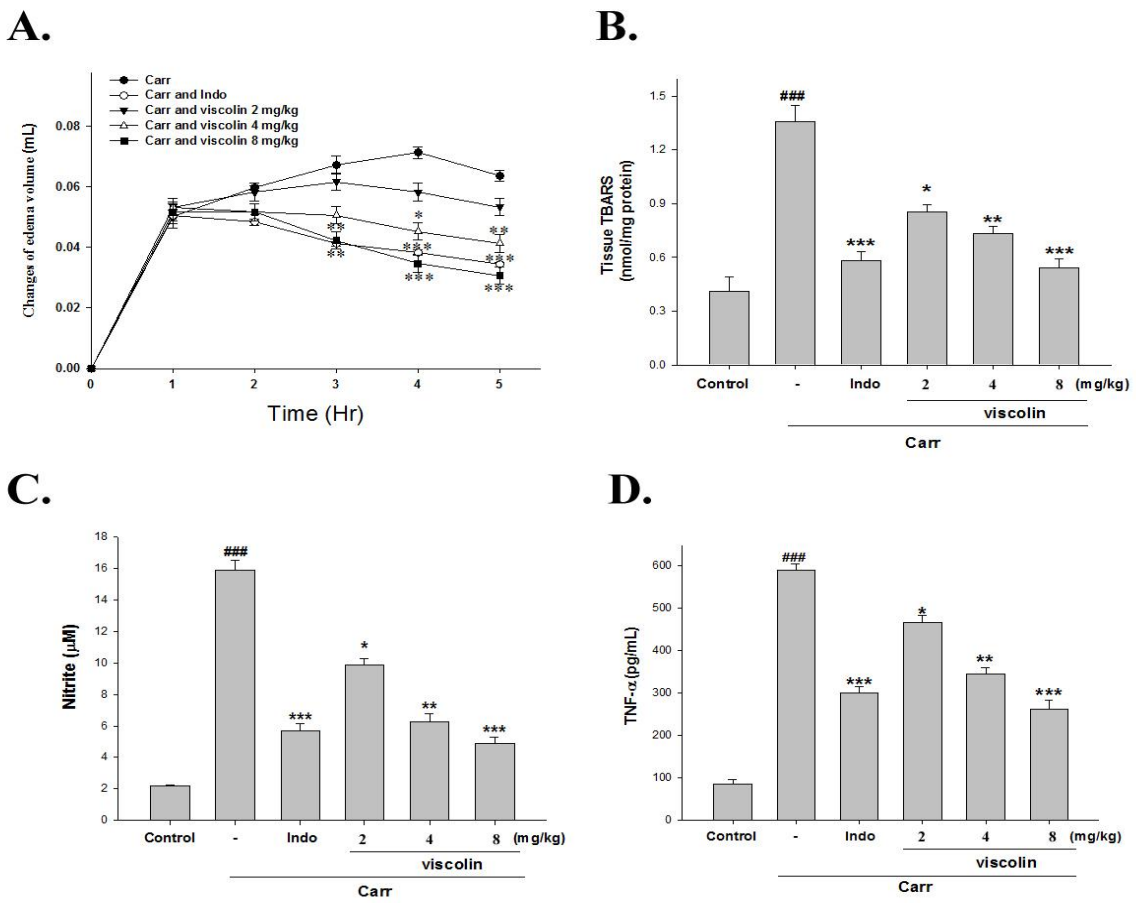
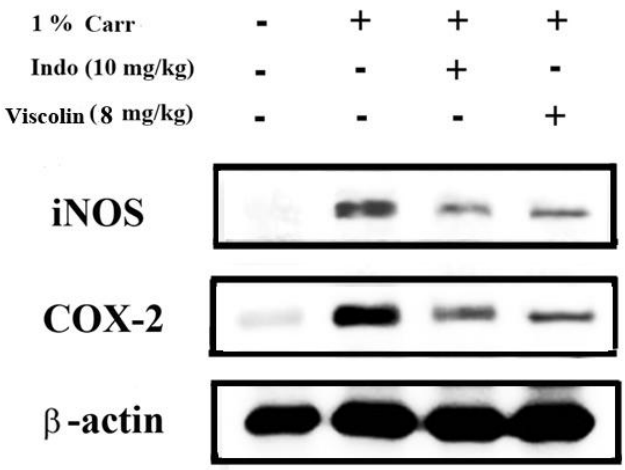
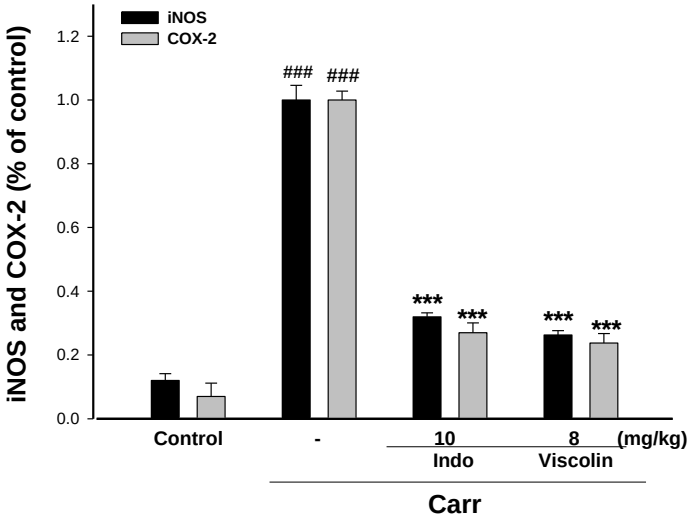


Figure 7. Effects of viscolin and Indo on hind paw edema induced by Carr in mice (A), the tissue MDA concentration of foot in mice (B), Carr-induced NO (C), and TNF-α (D) concentrations of serum at 5th hr in mice. Each value represents as mean ± S.E.M. $^{***}p < 0.001$ as compared with the Carr group (one-way ANOVA followed by Scheffe's multiple range test).



(A)



(B)

Figure 8. Inhibition of *i*NOS and COX-2 protein expressions by viscolin induced by Carr of foot at 5th hour in mice. (A) A representative Western blot from two separate experiments is shown. (B) Relative iNOS and COX-2 protein levels were calculated with reference to a Carr-injected mouse. ###compared with sample of control group. The data were presented as mean ± S.D. for three different experiments performed in triplicate. *** $p < 0.001$ were compared with Carr-alone group.

Figure 9.

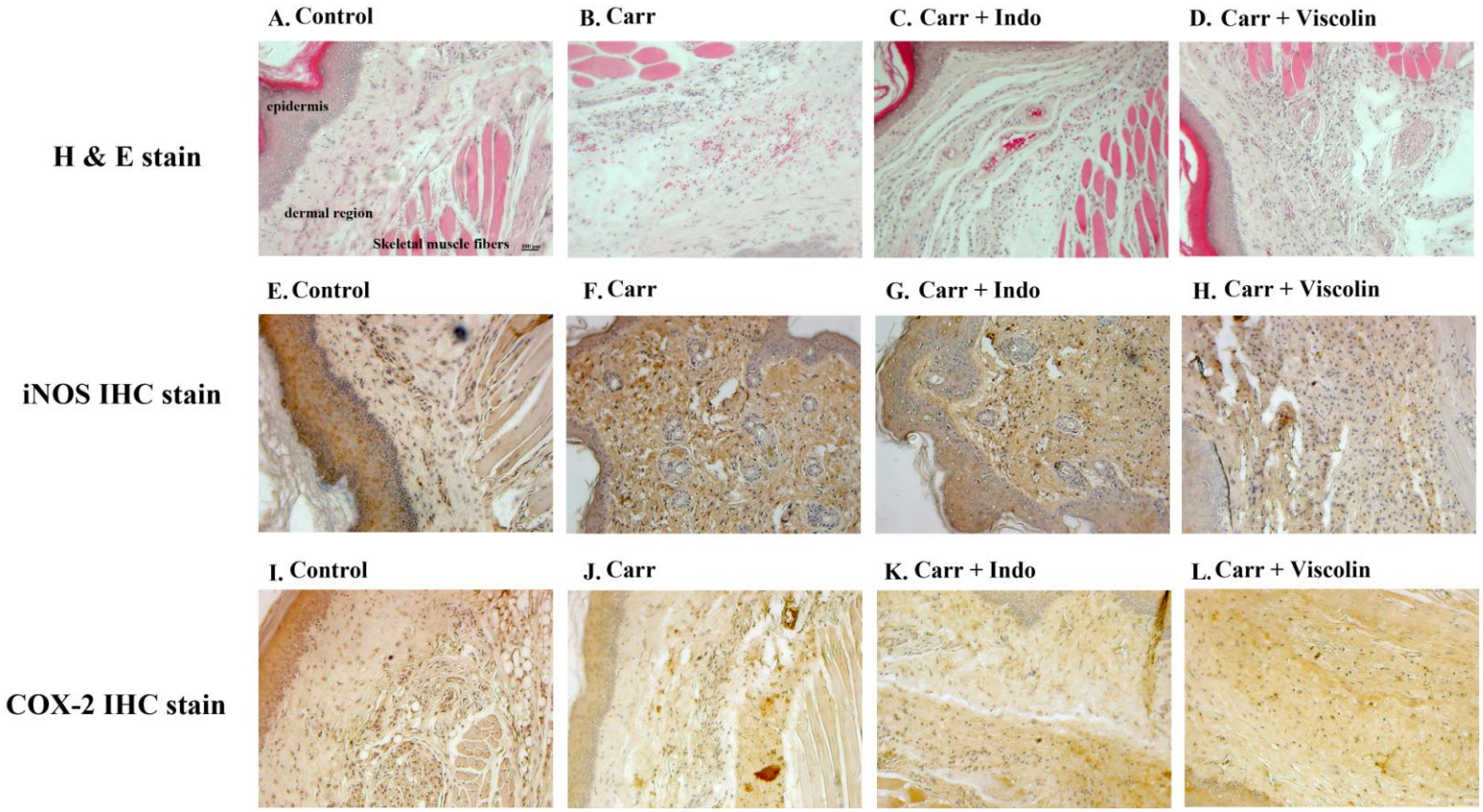

A. Control
B. Carr
C. Carr + Indo
D. Carr + Viscolin
H & E stain
epidermis
dermal region
Skeletal muscle fibers
E. Control
F. Carr
G. Carr + Indo
H. Carr + Viscolin
iNOS IHC stain
I. Control
J. Carr
K. Carr + Indo
L. Carr + Viscolin
COX-2 IHC stain

Figure 9. Histological appearances of mouse hind footpads after subcutaneously injecting 0.9% saline (Control group) or Carr, and then stained with H&E stain, while others were processed for iNOS and COX-2 immunohistochmistry staining. (A). Control mice: show the normal appearance of dermis and subdermis without any significant lesions, (F) iNOS and (J) COX-2 immunoreactive cells existed in the paws of normal mice; (B). Carr Only: Hemorrhage with moderately extravascular red blood cell and large amounts of inflammatory leucocytes, mainly neutrophils infiltrating the subdermis interstitial tissue. Moreover, detail of the subdermis layer show enlargement of the interstitial space caused by the exudate fluid in the edema, (G) numerous iNOS, and (K) COX-2 immunoreactive cells were observed in the brown site of paw tissue; (C). Carr + Indo 10mg/kg (i.p.) (100×): there were obvious morphological alterations and improvements, (H) iNOS and (L) COX-2 immunoreactive cells; (D). Carr + viscolin: there were significant morphological alterations compared to the tissue with Carr treatment only. The lesions showed no hemorrhage and the number of neutrophils infiltrating the subdermis interstitial tissue was markedly reduced and also in (I) iNOS and (M) COX-2 immunoreactive cells in paws. Moreover, no edema was seen in the interstitial space. (100×). The numbers of neutrophils were counted in each scope (400×) and thereafter obtain their average count from five scopes of every tissue slice. $^{###}p < 0.001$ as compared with the control group. $^{**}p < 0.01$, compared with Carr group.

Table 1. Effects of viscolin and indomethacin (Indo) on changes in CAT, SOD, and GPx activities in Carr-induced paw edema (5^{th} hour) 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	$3.54 \pm 0.15^{###}$	$1.65 \pm 0.13^{###}$	$7.21 \pm 0.07^{###}$
Carr+ Indo (10 mg/Kg)	$4.65 \pm 0.17^{**}$	$3.85 \pm 0.24^{**}$	$10.45 \pm 0.09^{**}$
Carr+ viscolin (2 mg/Kg)	$3.92 \pm 0.13^{*}$	$2.45 \pm 0.12^{*}$	$8.64 \pm 0.05^{*}$
Carr+ viscolin (4 mg/Kg)	$4.28 \pm 0.11^{**}$	$3.46 \pm 0.11^{**}$	$10.97 \pm 0.11^{**}$
Carr+ viscolin (8 mg/Kg)	$4.98 \pm 0.23^{***}$	$4.08 \pm 0.19^{***}$	$11.54 \pm 0.16^{***}$

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