Novel Aromatic Ester from *Piper longum* and Its Analogues Inhibit Expression of Cell Adhesion Molecules on Endothelial Cells†

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ABSTRACT: We report here the isolation and characterization of two active principles, ethyl 3′,4′,5′-trimethoxycinnamate (**1**) and piperine (**2**), from the combined hexane and chloroform extracts of *Piper longum*. Using primary human umbilical vein endothelial cells, we evaluated the activities of compound **1** on TNF-α-induced expression of cell adhesion molecules, viz., ICAM-1, VCAM-1, and E-selectin, which play key roles in controlling various inflammatory diseases. Both compounds **1** and **2** inhibited the TNF-α-induced expression of ICAM-1 in a dose- and time-dependent manner; however, the activity of ethyl 3′,4′,5′-trimethoxycinnamate (**1**) was ∼1.3 times higher than that of piperine (**2**). As ethyl 3′,4′,5′-trimethoxycinnamate (**1**) has been isolated for the first time from a natural source, *Piper longum*, and it exhibited higher activity, we carried out further studies on it. To correlate its cell adhesion molecule inhibitory activity with its functional consequences, we showed that it significantly blocked the adhesion of neutrophils to endothelium in a time- and concentration-dependent manner. Importantly, the inhibitory effect of cinnamate **1** was found to be reversible. To elucidate its structure−function−activity relationship, we synthesized nine different analogues of ethyl 3′,4′,5′-trimethoxycinnamate, i.e., compounds **3**−**11**, and compared the ICAM-1 inhibitory activity of compound **1** with those of its synthetic analogues as well as the corresponding acids **12**−**15**. The structure−activity studies indicate that the chain length of the alcohol moiety, substituents in the aromatic ring, and α, β-double bond of the cinnamic acid ester have significant effects on the inhibition of TNF-α-induced expression of ICAM-1 on endothelial cells. These findings have implications in developing compounds with a better therapeutic index against various inflammatory diseases.

Various inflammatory mediators, including cytokines such as TNF-α, IL-1β, and bacterial lipopolysaccharides, induce the expression of endothelial cell adhesion molecules, viz., intercellular adhesion molecule-1 (ICAM-1),[1] vascular cell adhesion molecule-1 (VCAM-1), and E-selectin, on the vascular endothelium (*1*). The increased level of expression of cell adhesion molecules on the endothelial cells alters the adhesive property of the vasculature, leading to indiscriminate infiltration of the leukocytes across the blood vessels, and hence causes inflammation (*2*). A critically regulated expression of these adhesion molecules is therefore highly essential for maintaining normal and healthy homeostasis in the body. A promising therapeutic approach for various inflammatory responses is to inhibit the cytokine-induced expression of cell adhesion molecules (*3*). Although various synthetic drugs, antibodies, and peptides have been demonstrated to inhibit the expression of these molecules, they have limitations for usage because of their side effects (*4*).

Several medicinal herbs have been shown to augment specific cellular and humoral immune response (*5*). *Piper*, an important genus of the family Piperaceae, comprises 700 species distributed in both hemispheres. This is a very large genus comprising creepers and shrubs, rarely herbs and trees, found throughout the tropical and subtropical regions of the world. *Piper* species are highly commercial, economical, and medicinally important (*6*). A large number of physiologically active compounds, viz., long chain esters and amides, alkaloids, lignans, neolignans, terpenes, steroids, chalcones, flavones, and flavanones, have been isolated from the genus *Piper* (*7*). *Piper longum* Linn., an important medicinal plant of the genus *Piper*, is used in traditional medicine by people in Asia and the Pacific Islands, and in particular in Indian traditional medicines (*8*). *P. longum* is a component of medicines reported to be used as a good remedy for treating gonorrhea, menstrual pain, tuberculosis, sleeping problems, respiratory tract infections, chronic gut-related pain, and arthritic conditions (*9*−*11*). Other reported beneficial effects of *P. longum* include analgesic and diuretic effects, relaxation of muscle tension, and alleviation of anxiety (*12*). Piperine

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[1] Abbreviations: ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; TNF-α, tumor necrosis factor α; IL-1β, interleukin-1β; NF-κB, nuclear factor κB; HUVECs, human umbilical vein endothelial cells.

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Table 1: ICAM-1 Inhibitory Activities

compound	concentration (μg/mL)[a]	% viability	% inhibition	IC_{50} (μg/mL)
DMSO (vehicle)	0.25%	97.2	none	none
ethyl 3′,4′,5′-trimethoxycinnamate (**1**)	50	95.4	90	25
piperine (**2**)	50	94.3	70	45
methyl 3′,4′,5′-trimethoxycinnamate (**3**)	130	91.5	80	100
propyl 3′,4′,5′-trimethoxycinnamate (**4**)	120	96.04	62	100
ethyl cinnamate (**5**)	130	95.95	85	95
propyl cinnamate (**6**)	140	95.08	70	100
isopropyl cinnamate (**7**)	100	95.44	70	85
butyl cinnamate (**8**)	140	94.99	55	125
ethyl 2′-hydroxy-3′-methoxycinnamate (**9**)	140	96.79	45	140[b]
propyl 2′-hydroxy-3′-methoxycinnamate (**10**)	90	95.14	40	90[b]
ethyl 3′,4′,5′-trimethoxydihydrocinnamate (**11**)	110	95.01	50	110
cinnamic acid (**12**)	140	95.19	50	110
2′-hydroxy-3′-methoxycinnamic acid (**13**)	140	96.75	55	125
3′,4′,5′-trimethoxycinnamic acid (**14**)	130	95.99	60	110
3′,4′,5′-trimethoxydihydocinnamic acid (**15**)	140	96.05	55	120

[a] Concentrations used where ~95% of the cells are viable. [b] These compounds did not reach up to 50% inhibition even at the maximum tolerable concentration.

was the first amide to be isolated from *Piper* species. It displays antipyretic, analgesic, insecticidal, and anti-inflammatory activities (*13*). Constituents of *Piper* species have inhibitory activity on prostaglandin and leukotriene synthesis in vitro (*14*). Further, piperine, one of the common constituents of *Piper*, has immunomodulatory and antitumor activity (*15*).

Although *P. longum* and its constituents have been implicated in many different pathological conditions, no study involving them in the inhibition of expression of cell adhesion molecules has been reported. Recently, we have reported the ICAM-1 inhibitory activity of three extracts of *Piper* species (*16*). The study presented here involves the isolation and characterization of a novel cinnamic acid ester, viz., ethyl 3′,4′,5′-trimethoxycinnamate (**1**), and piperine **2** from the combined active hexane and chloroform extracts of *P. longum* and reports their inhibitory effect on TNF-α-induced expression of cell adhesion molecules on human umbilical vein endothelial cells. Further, nine different analogues of natural cinnamate **1**, i.e., compounds **3**−**11**, have been synthesized, and the structure−activity relationship has been studied on the basis of the activity of natural and synthetic cinnamates and their corresponding acids **12**−**15**.

MATERIALS AND METHODS

Materials. Anti-ICAM-1, anti-E-selectin, anti-VCAM-1, and TNF-α were purchased from Pharmingen. M-199 medium, L-glutamine, endothelial cell growth factor, trypsin, Pucks saline, HEPES, *o*-phenylenediamine, and anti-mouse IgG-bound HRP were purchased from Sigma Chemical Co. Fetal calf serum was purchased from Biological Industries.

Cinnamic acid (**12**), 2-hydroxy-3-methoxycinnamic acid (**13**), 3,4,5-trimethoxycinnamic acid (**14**), and 3,4,5-trimethoxydihydrocinnamic acid (**15**) were purchased from Aldrich Chemical Co.

Cells and Cell Culture. Primary endothelial cells were isolated from human umbilical cord using mild trypsinization (*17*). The cells were grown in M-199 medium supplemented with 15% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, endothelial cell growth factor (50 μg/mL), and heparin (5 units/mL). At confluence, the cells were subcultured using a 0.05% trypsin/0.01 M EDTA solution and were used between passages three and four. The viability of cells was determined by a trypan blue exclusion test. The purity of endothelial cells was determined by E-selectin expression.

Cell Viability Assay. The cytotoxicity of compound **1** and its analogues was analyzed by using the trypan blue exclusion test as described previously (*18*), and it was further confirmed by a colorimetric MTT (methylthiazolyldiphenyltetrazolium bromide) assay as described previously (*19*). Briefly, endothelial cells were plated onto 96-microwell plates and allowed to attach overnight. The next day, the cells were treated with DMSO alone (0.25%, as vehicle) or with different concentrations of compound **1** or its analogues for 24 h. Four hours before the end of the incubation, medium was removed and 100 μL of MTT (5 mg/mL in PBS) was added to each well. The MTT was removed after 4 h; cells were washed out with PBS, and 100 μL of DMSO was added to each well to dissolve water insoluble MTT−formazan crystals. Absorbance was recorded at 570 nm in an ELISA reader (Bio-Rad, model 680). All experiments were performed at least three times in triplicate wells. DMSO was used as a solvent (vehicle) for dissolving all the compounds that were tested. The DMSO concentration (0.25%) was held constant in all the experiments, and this low concentration does not cause any cytotoxicity on endothelial cells (Table 1).

Modified ELISA for Measurement of ICAM-1 and E-Selectin. A cell-ELISA was used to assess the expression of ICAM-1 and E-selectin on the surface of endothelial cells (*18*). Endothelial cells were incubated with or without the test compound at desired concentrations for the required period followed by treatment with TNF-α (10 ng/mL) or LPS (1 μg/mL) for 16 h for ICAM-1 expression and 4 h for E-selectin expression. The cells were fixed with 1.0% glutaraldehyde and using nonfat dry milk (3.0% in PBS-blocked nonspecific binding of antibody). Following incubation overnight at 4 °C with ICAM-1 and E-selectin mAb, diluted in blocking buffer, the cells were washed with PBS and incubated with peroxidase-conjugated goat anti-mouse secondary Ab. The cells were again washed with PBS and exposed to the peroxidase substrate [40 mg of *o*-phenylenediamine dihydrochloride/100 mL in citrate phosphate buffer

Table 2: Melting Points and HRMS Data of Known Compounds

compound	yield (%)	mp (°C)		HRMS (EI) $[M]^+$ peak	
		literature	observed	calculated	observed
methyl 3′,4′,5′-trimethoxycinnamate (**3**)	97	95−98 (*29*)	97	252.0998	252.0985
ethyl cinnamate (**5**)	95	oil (*22*, *24*)	oil	176.0837	176.0834
propyl cinnamate (**6**)	93	oil (*22*, *25*)	oil	190.0994	190.0988
isopropyl cinnamate (**7**)	90	oil (*22*, *26*)	oil	190.0994	190.0991
butyl cinnamate (**8**)	96	oil (*22*, *25*)	oil	204.1150	204.1160
ethyl 2′-hydroxy-3′-methoxycinnamate (**9**)	89	68−69 (*27*)	68	222.0892	222.0902
ethyl 3′,4′,5′-trimethoxydihydrocinnamate (**11**)	95	102−103 (*28*)	102	268.1311	268.1301

(pH 4.5)]. The color development reaction was stopped by the addition of 2 N sulfuric acid, and absorbance at 490 nm (A_{490}) was measured using an automated microplate reader (Spectramax 190, Molecular Devices). The A_{490} for each control at each concentration of the compound was set to unity, and the change in the relative level of induction was calculated. The percentage inhibition was calculated as [1 − A_{490} (increasing amount of compound)/A_{490} (control)] × 100.

Flow Cytometry Analysis. The cell surface expression of ICAM-1, VCAM-1, and E-selectin on endothelial cells was analyzed by flow cytometry (*18*). The endothelial cells were incubated with or without compound **1** for 1 h. The cells were further treated with TNF-α (10 ng/mL) and incubated for 16 h for ICAM-1 and VCAM-1, and for 4 h for E-selectin expression. The cells were washed with PBS and dislodged, after which they were incubated with anti-ICAM-1, anti-VCAM-1, and anti-E-selectin antibodies (1.0 μg/10^6 cells, 30 min, 4 °C). After incubation, the cells were washed with PBS and then stained with FITC-conjugated goat anti-mouse IgG for 30 min at 4 °C. The cells were then fixed with 1.0% paraformaldehyde and were analyzed to estimate the level of expression of cell adhesion molecules using a flow cytometer (FACSVantage, Becton & Dickinson). For each sample, 20 000 events were recorded. Analysis was carried out by using Cell Quest (Becton & Dickinson). The autofluorescence intensity was subtracted from that under treated conditions; the mean fluorescence intensity was estimated from three independent experiments, and bar diagrams were plotted.

Neutrophil Isolation. Neutrophils were isolated from peripheral blood of healthy individuals (*18*). Blood was collected in a heparin solution (20 units/mL), and erythrocytes were removed by sedimentation against a 6% dextran solution. Plasma, rich in white blood cells, was layered over a Ficoll-hypaque solution followed by centrifugation (300*g* for 20 min at 20 °C). The top saline layer and the Ficoll-hypaque layer were aspirated, leaving a neutrophil−RBC pellet. The residual red blood cells were removed by hypotonic lysis. Isolated cells were washed with PBS and resuspended in PBS containing 5 mM glucose, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ at a final concentration of 6×10^5 cells/mL. This procedure usually resulted in approximately 95% neutrophils, and the cell viability was more than 95% as detected by a trypan blue exclusion test.

Cell Adhesion Assay. Neutrophil adhesion assays were performed under static conditions as described previously (*18*). Endothelial cells plated in 96-well culture plates were incubated with or without compound **1** at desired concentrations for 1 h, followed by induction with TNF-α (10 ng/mL) for 6 h. Endothelial monolayers were washed with PBS, and neutrophils (6×10^4 per well) were added over them and were allowed to adhere for 1 h at 37 °C. The nonadherent neutrophils were washed with PBS, and neutrophils bound to endothelial cells were assayed by adding a substrate solution consisting of *o*-phenylenediamine dihydrochloride [40 mg/100 mL in citrate phosphate buffer (pH 4.5)], 0.1% cetitrimethylammonium bromide, and 3-amino-1,2,4-triazole (1 mM). The absorbance was read at 490 nm using an automated microplate reader (model 680, Bio-Rad). The percent inhibition of neutrophil adhesion was calculated as mentioned in the modified ELISA.

Extraction of P. longum Fruits and Isolation and Identification of Compounds from the Extract. P. longum fruit powder (100 g) was extracted with 50% aqueous ethanol (150 mL). The supernatant (140 mL) collected by centrifugation at 14 000 rpm was dried in a vacuum and designated as an ethanolic extract. This was further fractionated using *n*-hexane (35 mL). The hexane layer was dried and designated as a hexane fraction. The residual material was fractionated with chloroform (40 mL) and designated as a chloroform extract. The hexane and chloroform extracts of fruits of *P. longum* were combined, because they exhibited almost similar spots on TLC examination. The combined extract was purified by column chromatography over silica gel using a gradient mixture of ethyl acetate and petroleum ether as the eluent; ethyl 3′,4′,5′-trimethoxycinnamate (**1**) and piperine (**2**) were eluted with 5 and 17% ethyl acetate/petroleum ether solutions, respectively. The structures of both compounds **1** and **2** were established on the basis of their spectral analysis (IR, ^{1}H NMR, and ^{13}C NMR spectra and HRMS) and confirmed by comparison of spectral data and melting points with those reported in the literature (*20*, *21*). Compound **1** has been isolated for the first time from a natural source. It was previously synthesized by Tanoguchi el al. (*20*) as a precursor for the synthesis of a natural product; the authors have reported only ^{1}H NMR and partial IR spectral data of the compound. We herein report the complete IR, ^{1}H NMR, and ^{13}C NMR spectral data of compounds **1** and **2**, the HRMS data of known compounds in Table 2**,** and complete spectra of novel compounds **4** and **10** in the Supporting Information.

Ethyl 3′,4′,5′-trimethoxycinnamate (1) was obtained as a colorless crystalline solid (80 mg): mp 60−62 °C [lit. (*20*) mp 67 °C]; $R_f = 0.25$ (0.5% methanol/chloroform); IR (KBr) 3001, 2975, 2946, 2837, 1702 (CO), 1633, 1583, 1505, 1471, 1453, 1416, 1313, 1280, 1244, 1178, 1122, 997, 983, 824 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.34 (3H, t, $J = 7.5$ Hz, CH_3), 3.88 (9H, s, 3 × OCH_3), 4.25 (2H, q, $J = 7.5$ Hz, OCH_2), 6.35 (1H, d, $J = 15.9$ Hz, C-2H), 6.75 (2H, s, C-2′H and C-6′H), 7.59 (1H, d, $J = 15.9$ Hz, C-3H); ^{13}C NMR (300 MHz, $CDCl_3$) δ 14.69 (CH_3), 56.54 (C-3′ and C-5′

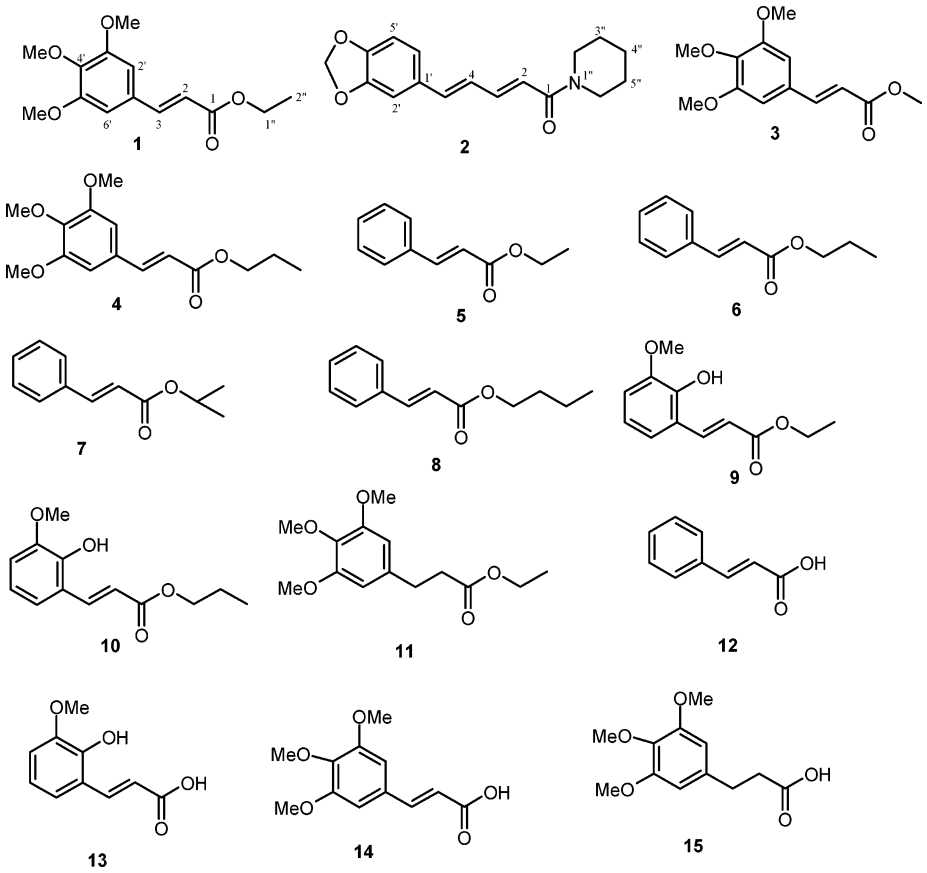

FIGURE 1: Structures of compounds that were tested.

OCH_3), 60.84 (C-4′ OCH_3), 61.30 (OCH_2), 105.50 (C-2), 117.92 (C-2′ and C-6′), 130.35 (C-1′), 144.91 (C-3), 145.20 (C-4′), 153.83 (C-3′ and C-5′), 167.88 (C-1); HRMS calcd for $C_{14}H_{18}O_5$ *m*/*z* 266.1154, observed *m*/*z* 266.1165.

*Piperine (**2**)* was obtained as a colorless crystalline solid (80 mg): mp 125−128 °C [lit. (*21*) mp 128−129 °C]; R_f = 0.43 (40% ethyl acetate/petroleum ether); IR (KBr) 2940, 1634 (CO), 1611, 1584, 1491, 1447, 1252, 1193, 1133, 1031, 996, 927, 847, 830, 804, 717, 608 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.62 (4H, m, C-3″H and C-5″H), 1.66 (2H, m, C-4″H), 3.58 (4H, m, C-2″H and C-6″H), 5.97 (2H, s, OCH_2O), 6.43 (1H, d, J = 14.7 Hz, C-2H), 6.74 (2H, m, C-3H and C-4H), 6.75 (1H, d, J = 8.00 Hz, C-5′H), 6.89 (1H, dd, J = 8.00 and 1.34 Hz, C-6′H), 6.97 (1H, d, J = 1.34 Hz, C-2′H), 7.40 (1H, m, C-5H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 25.04, 26.01 and 27.08 (C-3″ and C-4″ and C-5″), 43.62 and 47.25 (C-2″ and C-6″), 101.62 (OCH_2O), 106.08 (C-2), 108.84 (C-3), 120.51, 122.80, 125.78 (C-2′, C-5′, and C-6′), 131.44 (C-1′), 138.53 (C-4), 142.77 (C-5), 148.49 and 148.58 (C-3′ and C-4′), 165.81 (C-1).

General Method of Preparation of Analogues of Natural Cinnamic Acid Ester. Nine different analogues of natural cinnamic acid ester **1**, i.e., compounds **3**−**11**, have been synthesized in quantitative yields by refluxing the mixture of different cinnamic acids with the corresponding alcohol in the presence of a catalytic amount of sulfuric acid following the known methodology (*22*, *23*) (Figure 1; see the Supporting Information).

Statistical Analysis. Results are given as means ± the standard deviation. An independent two-tailed Student's *t* test was performed. Differences were considered statistically significant if $p < 0.05$. All statistical analysis was performed by using Microcal Origin version 3.0 (Microcal Software Inc., Northampton, MA). Flow cytometry data were analyzed by using Cell Quest (Becton & Dickinson).

RESULTS

*Ethyl 3′,4′,5′-Trimethoxycinnamate (**1**) and Its Analogues Are Not Toxic to Endothelial Cells.* To evaluate the effect of these compounds on the viability of endothelial cells, cells were incubated with or without these compounds and tested for viability by the trypan blue exclusion test (data not shown) and the MTT assay as described in Materials and Methods. As shown in Table 1, the concentrations of the compounds were chosen where ∼95% of the cells were viable for further experiments.

*Ethyl 3′,4′,5′-Trimethoxycinnamate (**1**) and Piperine (**2**) Inhibit TNF-α-Induced Expression of ICAM-1.* To examine the effect of ethyl 3′,4′,5′-trimethoxycinnamate (**1**) and piperine (**2**) on TNF-α-induced expression of ICAM-1, HUVECs were incubated with or without cinnamate **1** or piperine (**2**) at various concentrations for 2 h prior to induction with TNF-α (10 ng/mL) for 16 h. As detected by the cell-ELISA, ICAM-1 was expressed at low levels on unstimulated endothelial cells and was induced almost 3-fold by stimulation with TNF-α. Interestingly, treatment of cells with ethyl 3′,4′,5′-trimethoxycinnamate (**1**) led to a significant reduction in the level of TNF-α-induced expression of ICAM-1 in a concentration-dependent manner (Figure 2). The extent of inhibition of TNF-α-induced expression of ICAM-1 was approximately 90%. Similarly, piperine (**2**) led to a significant reduction in the level of TNF-α-induced

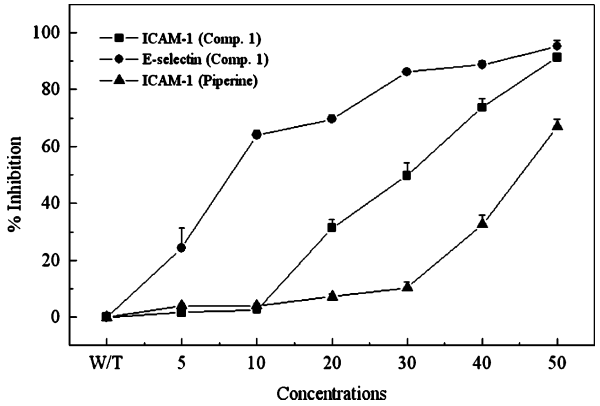

FIGURE 2: Concentration-dependent inhibition of TNF-α-induced ICAM-1 and E-selectin expression by compound **1** and piperine. Endothelial cells were grown to confluence in 96-well plates and incubated with or without (W/T) indicated concentrations of the compound for 2 h prior to induction with TNF-α (10 ng/mL) for 16 h for ICAM-1 and 4 h for E-selectin. The ICAM-1 and E-selectin level on the cells was measured by the ELISA as described in Materials and Methods. The data presented are representative of three independent experiments. Values are means ± the standard deviation of quadruplicate wells. The data for the inhibition of ICAM-1 by compound **1** and piperine and the inhibition of E-selectin by compound **1** are shown with the symbols ■, ▲, and ●, respectively.

expression of ICAM-1 in a concentration-dependent manner (Figure 2). The level of inhibition of TNF-α-induced expression of ICAM-1 was approximately 70%. It has, however, been observed that both compound **1** and piperine (**2**) caused a slight increase at lower concentrations and a slight decrease at higher concentrations in the basal level of ICAM-1 expression (data not shown). The inhibition by 3′,4′,5′-trimethoxycinnamate **1** and piperine (**2**) remains unchanged if HUVECs were stimulated with LPS instead of TNF-α (data not shown).

Ethyl 3′,4′,5′-Trimethoxycinnamate (1) Inhibits TNF-α-Induced Expression of E-Selectin and VCAM-1. To examine the effect of ethyl 3′,4′,5′-trimethoxycinnamate (**1**) on TNF-α-induced expression of E-selectin, HUVECs were incubated with or without it at various concentrations for 2 h prior to induction with TNF-α (10 ng/mL) for 4 h. As detected with the cell-ELISA, E-selectin was expressed at low levels on unstimulated endothelial cells and was induced ∼3-fold by stimulation of TNF-α. It had no effects on the basal level of E-selectin expression, whereas it led to a significant reduction in the level of TNF-α-induced expression of E-selectin in a concentration-dependent manner (Figure 2). The level of inhibition of TNF-α-induced expression of E-selectin was approximately 95%.

The inhibitory activity of ethyl 3′,4′,5′-trimethoxycinnamate on ICAM-1 and E-selectin expression was further confirmed by flow cytometry (Figure 3A, C). In addition to ICAM-1 and E-selectin, we also examined the VCAM-1 inhibition on endothelial cells by compound ethyl 3′,4′,5′-trimethoxycinnamate **(1)**. The unstimulated cells expressed low levels of ICAM-1, VCAM-1, and E-selectin, and upon stimulation with TNF-α, a substantial increase (6−8-fold) in the level of expression of all these three molecules was observed (Figure 3A−C). Pretreatment of endothelial cells with ethyl 3′,4′,5′-trimethoxycinnamate (50 μg/mL) significantly inhibited TNF-α-induced expression of ICAM-1, VCAM-1, and E-selectin by >90% (Figure 3A−C). Thus,

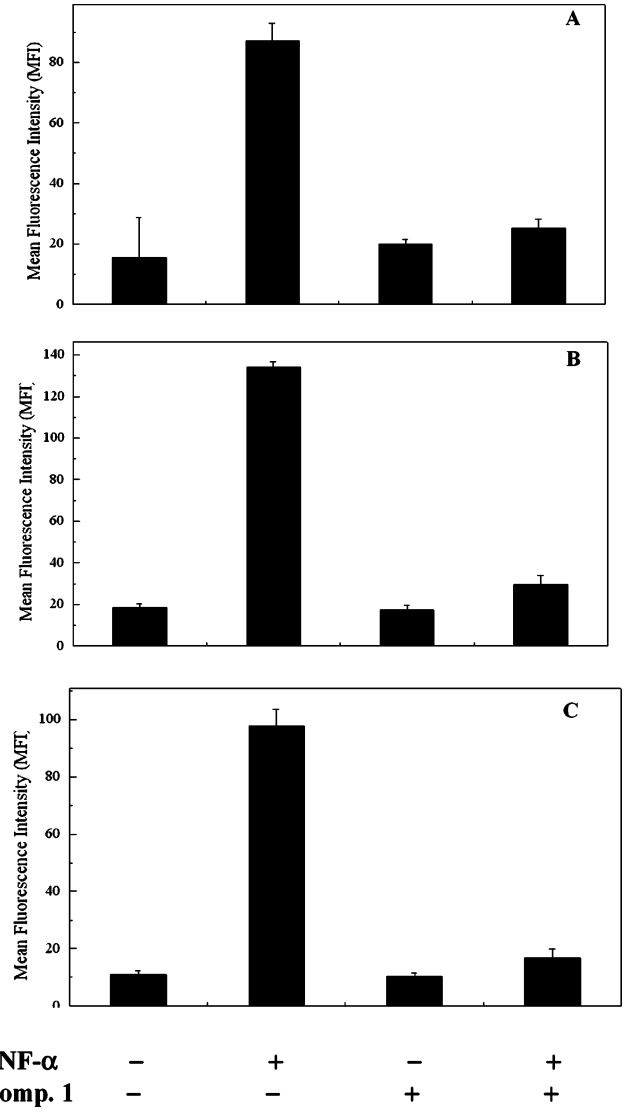

FIGURE 3: Flow cytometry analysis of inhibition of TNF-α-induced ICAM-1, VCAM-1, and E-selectin expression by compound **1**. The endothelial cells were treated with 50 μg/mL ethyl 3′,4′,5′-trimethoxycinnamate (**1**) for 1 h followed by stimulation with TNF-α (10 ng/mL) for 16 h for VCAM-1 and ICAM-1 and for 4 h for E-selectin. Expression of these molecules was assessed by flow cytometry as described in Materials and Methods. The data are means ± the standard deviation of three independent experiments after autofluorescence was subtracted from values for treated conditions. Cell Quest was used for statistical analysis ($p < 0.05$): (A) ICAM-1, (B) VCAM-1, and (C) E-selectin.

ethyl 3′,4′,5′-trimethoxycinnamate inhibits the induced expression of cell adhesion molecules as measured using the cell-ELISA and confirmed by flow cytometry.

Kinetics of Inhibition by Ethyl 3′,4′,5′-Trimethoxycinnamate (1). To study the kinetics of inhibition by ethyl 3′,4′,5′-trimethoxycinnamate (**1**), the cells were treated with compound **1** at 40 μg/mL for various time points, and then the pretreatment and posttreatment effects of this compound were studied. We found that only pretreatment of cells with ethyl 3′,4′,5′-trimethoxycinnamate (**1**) inhibited the expression of ICAM-1 on endothelial cells, whereas posttreatment had no significant effect (Figure 4). This observation indicates that ethyl 3′,4′,5′-trimethoxycinnamate (**1**) interferes with some earlier steps of the signaling cascade for inhibiting the TNF-α-induced expression of cell adhesion molecules.

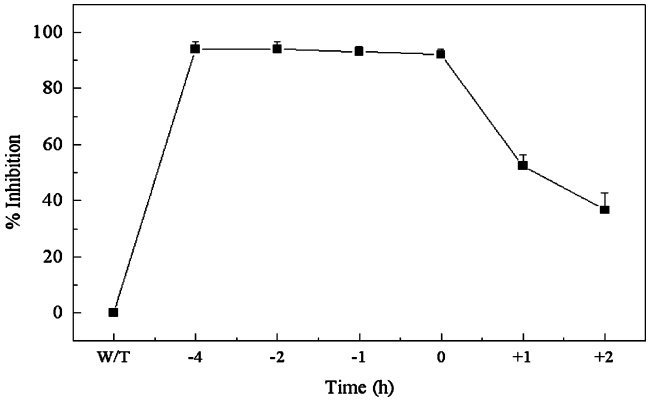

FIGURE 4: Kinetics of inhibition by compound **1**. Endothelial cells grown to confluence in 96-well plates were preincubated with or without (W/T) 40 μg/mL compound **1** for the indicated time periods (represented as negative time points prior to induction with TNF-α). Induction was with TNF-α (10 ng/mL**)** before and after treatment of the cells with the compound at given time points. The ICAM-1 level on the cells was measured by the cell-ELISA as described in Materials and Methods. The data are representative of three independent experiments. Values are means ± the standard deviation of quadruplicate wells.

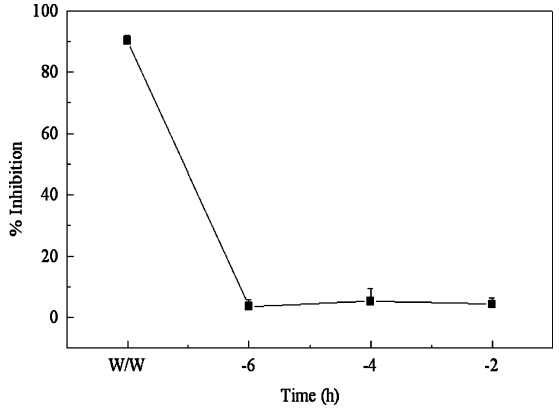

FIGURE 5: Inhibition by compound **1** is reversible. Endothelial cells grown to confluence in 96-well plates were incubated with or without 40 μg/mL compound **1** for the indicated time periods. After being washed or not being washed (W/W) with medium twice, the cells were incubated in complete medium for 1 h. This was followed by induction with TNF-α (10 ng/mL) for 16 h. The ICAM-1 level on the cells was measured by the cell-ELISA as described in Materials and Methods. The data are representative of three independent experiments. Values are means ± the standard deviation of quadruplicate wells.

*Inhibition by Ethyl 3′,4′,5′-Trimethoxycinnamate (**1**) Is Reversible.* To study whether ethyl 3′,4′,5′-trimethoxycinnamate (**1**) causes any permanent change in the endothelial cells, they were preincubated with compound **1** (40 μg/mL) for varying amounts of time ranging from 2 to 6 h, washed, and allowed to recover for 1 h, followed by induction with TNF-α (10 ng/mL) for 16 h. As detected by the ELISA, the effect of compound **1** was found to be reversible as the cells were capable of responding to TNF-α, and no permanent change was observed upon treatment with the compound **1** (Figure 5).

*Ethyl 3′,4′,5′-Trimethoxycinnamate (**1**) Inhibits Adhesion of Neutrophils to Endothelium.* It is well established that ICAM-1, VCAM-1, and E-selectin play a major role in the adhesion of neutrophils to endothelium. To examine the functionality of ethyl 3′,4′,5′-trimethoxycinnamate (**1**) on the adhesion of neutrophils to endothelium, HUVECs were

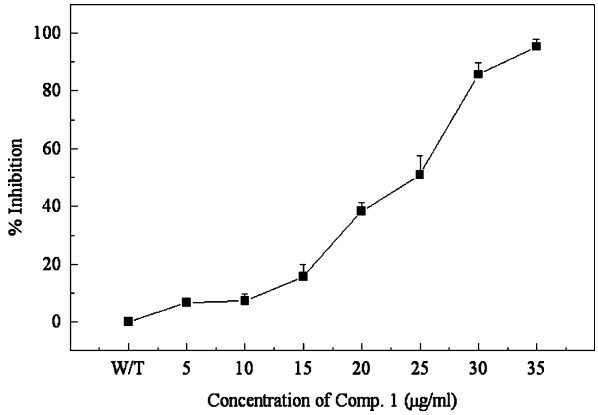

FIGURE 6: Inhibition of adhesion of neutrophils to endothelium by compound **1**. Endothelial cells were grown to confluence in 96-well plates and incubated with or without (W/T) the indicated concentrations of compound **1** for 2 h prior to induction with TNF-α (10 ng/mL) for 6 h. The adhesion of neutrophils on the cells was assessed by the colorimetric assay as described in Materials and Methods. The data are representative of three independent experiments. Values are means ± the standard deviation of quadruplicate wells.

incubated with or without it at various concentrations for 2 h prior to induction with TNF-α (10 ng/mL) for 6 h. As detected by the colorimetric assay, there was weak adherence of neutrophils on unstimulated endothelial cells, and adherence was induced more than 3-fold by stimulation with TNF-α. Interestingly, 3′,4′,5′-trimethoxycinnamate (**1**) significantly inhibited the adhesion of neutrophils to endothelium in a concentration-dependent manner, and the maximum level of inhibition was approximately 90% (Figure 6).

*Analogues of Natural Cinnamate **1**, i.e., Compounds **3**−**11**, and Their Corresponding acids **12**−**15**, Inhibit TNF-α-Induced Expression of ICAM-1.* To elucidate the structure−activity relationship, nine different analogues of natural cinnamate **1**, i.e., compounds **3**−**11**, that differ either in the alcohol moiety of the ester or in the substituents on the phenyl ring and are derived from the dihydro derivative of cinnamic acid were synthesized as described in Materials and Methods. The analogues were selected with a view of identifying important structural components in ethyl 3′,4′,5′-trimethoxycinnamate (**1**) that are required for its high inhibitory activity.

These analogues were evaluated at various concentrations for their effects on inhibition of TNF-α-induced expression of ICAM-1 on endothelial cells (Table 1). Different levels of inhibition (percent) of the synthetic cinnamates **3**−**11** and their corresponding acids **12**−**15** were compared with the inhibitory activity of ethyl 3′,4′,5′-trimethoxycinnamate (**1**) and piperine (**2**). The IC_{50} of each compound was calculated separately from their respective activity−concentration graphs (Table 1). Also, the levels of inhibition (percent) were shown at the maximum tolerable concentration where cell viability and morphology were not affected (Table 1).

DISCUSSION

The main objective of this study was to focus on the isolation and characterization of the novel compound from the active extracts of *P. longum* and the evaluation of the potential of pure natural isolates for inhibition of TNF-α-induced expression of ICAM-1 on endothelial cells with an

aim of identifying the active constituents. Immunoregulation is a complex balance between regulatory and effector cells, and any imbalance in immunological mechanism can lead to pathogenesis (*30*). Cell adhesion molecules play critical roles in the recruitment and migration of cells to sites of inflammation. Not surprisingly, these receptors have garnered the attention of the pharmaceutical industry as targets for the development of drugs to treat inflammatory and autoimmune diseases (*31*, *32*). The effects of many anti-inflammatory drugs can be ascribed, in part, to inhibition of the expression of CAMs. However, in the search for more selective and potent drugs for clinically important diseases, such as multiple sclerosis, asthma, rheumatoid arthritis, inflammatory bowel disease, allergies, and atherosclerosis, direct inhibition of the functions of CAMs has attracted growing interest.

Herein, we for the first time report the ICAM-1 inhibitory activity of ethyl 3′,4′,5′-trimethoxycinnamate (**1**) and piperine (**2**) isolated from combined active hexane and chloroform extracts of *P. longum*, analogues of cinnamate **1**, i.e., compounds **3**−**11**, and their corresponding acids **12**−**15** (Figure 1 and Table 1). The dosages of tested compounds were selected on the basis of their maximum concentration tolerated by the cells and maximum activity. The results of TNF-α-induced expression of ICAM-1 inhibitory activity of natural ethyl 3′,4′,5′-trimethoxycinnamate (**1**), piperine (**2**), analogues of natural cinnamate, **3**−**11**, and their corresponding acids **12**−**15** revealed that natural cinnamate **1** is the most active candidate, which inhibits the expression of ICAM-1 by 90% at a concentration of 50 μg/mL (Table 1). In addition to ICAM-1, compound **1** also significantly inhibited TNF-α-induced expression of VCAM-1 and E-selectin. It has been noticed that the basal levels of expression of ICAM-1, but not VCAM-1 or E-selectin, were slightly increased at lower concentrations and slightly decreased at higher concentrations of compound **1** (data not shown). Although the reason behind this alteration is not clear, the effect is not statistically significant. Also, this is not due to the cytotoxicity of compound **1**, as the basal levels of expression of VCAM-1 and E-selectin were not altered.

The functionality of inhibition of cell adhesion molecules by ethyl 3′,4′,5′-trimethoxycinnamate (**1**) on the adhesion of neutrophils to endothelium was also demonstrated in our studies. However, the IC_{50} for suppression of neutrophil adhesion (∼100 μM) was found to be higher than the IC_{50} of E-selectin (∼30 μM) and similar to the IC_{50} of ICAM-1 (100 μM). This observation is not surprising, as there exists a hierarchy for the interactions of cell adhesion molecules on endothelial cells for the adhesion of leukocytes to the endothelial monolayer (*33*).

The IC_{50} value of cinnamate **1** is 25 μg/mL. Although the maximum tolerable concentration of other natural isolate, piperine (**2**), is the same as that of cinnamate **1**, its inhibitory activity is almost 1.3 times lower than the activity of the cinnamate. Thus, piperine **2** exhibited 70% inhibition of TNF-α-induced expression of ICAM-1 compared to 90% inhibition by cinnamate **1**. Further, the IC_{50} value of cinnamate is 1.8 times lower than the IC_{50} value of piperine. This suggests that the presence of an amide bond on the test compound is unfavorable and the presence of an ester bond is favorable.

The comparison of the activity of natural cinnamate **1** with its synthetic analogues **3**−**11** indicates that the chain length of the alcohol moiety of cinnamic acid esters has a significant effect on inhibition of TNF-α-induced expression of ICAM-1 on endothelial cells (Table 1). Thus, the change in the alcohol moiety of compound **1** from ethyl to methyl and propyl in compounds **3** and **4** decreases the activity from 90 to 80% and from 90 to 62%, respectively. This change in the length of the alcohol moiety also results in increases in IC_{50} from 25 to 100 μg/mL. The decrease in the activity of cinnamic acid ester with the increase in chain length is supported by the fact that the activities of ethyl, propyl, and butyl cinnamates **5**, **6**, and **8** decrease in order (Table 1). Thus, compounds **5** and **6** exhibited 85 and 70% inhibition of TNF-α-induced expression of ICAM-1, respectively; i.e., compound **5** having an alcohol moiety one CH_2 shorter is 1.2 times more active than compound **6**, which in turn is almost 1.3 times more active than compound **8** which has one more CH_2 unit in its alcohol moiety. This decrease in the activity with an increase in the chain length of the alcohol moiety in the ester proves the earlier hypothesis (*34*). Branching of the chain of the alcohol moiety of cinnamate makes the compound more active. Thus, the IC_{50} value of cinnamate **7** (85 μg/mL) is lower than that of its isomeric compound **6** (100 μg/mL), because the alcohol moiety of this compound is branched (Table 1).

The comparison of the activity of ethyl trimethoxycinnamate **1** with the activities of ethyl and propyl 2-hydroxy-3-methoxycinnamates **9** and **10**, respectively, reveals that the change in the methoxy group present on the aromatic ring of the cinnamic acid moiety to a hydroxy group drastically reduces the activity. Thus, the activity of cinnamate **1** is 2 and 2.25 times greater than the activities of compounds **9** and **10**, respectively. It is not only the decrease in the percent inhibitory activity but also the maximum tolerable concentration of the hydroxy esters **9** and **10** that is much higher than the concentration limit for methoxy cinnamate **1**. Thus, the maximum tolerable concentrations of hydroxy esters **9** and **10** are 140 and 90 μg/mL, respectively, whereas the limit for trimethoxycinnamate **1** is only 50 μg/mL. In fact, compounds **9** and **10** did not reach 50% inhibition even at the maximum tolerable concentration. It has also been observed that the replacement of the cinnamic acid moiety of the ester with dihydrocinnamic acid reduces the activity of the compound for inhibition of TNF-α-induced expression of ICAM-1. Thus, the activity of trimethoxycinnamate **1** is 1.8 times higher than the activity of its dihydro analogue trimethoxydihydrocinnamate **11**. The reduction of the cinnamic acid moiety also increases the toxicity of the compound by a factor of 2.2. The activities of the corresponding cinnamic acids **12**−**15** of the esters **3**−**11** were also evaluated to determine their relative potential of inhibition of TNF-α-induced expression of ICAM-1. It has been found that the IC_{50} values of all four cinnamic acids, **12**−**15**, were 4.4−5.0 times higher than the IC_{50} value of the most active natural cinnamate, **1** (Table 1).

The comparison of the activity of inhibition of TNF-α-induced expression of ICAM-1 on endothelial cells by natural cinnamate **1** and piperine **2** and those of ester analogues **3**−**11** and corresponding cinnamic acids **12**−**15** of natural and synthetic cinnamates indicates that the double bond and methoxy substituents in the cinnamate moiety are essential

for biological activity. Further, the chain length and skeleton of the alcohol moiety of the cinnamate ester are also important for the inhibition of TNF-α-induced expression of ICAM-1; ethyl esters have proved to be the best choice. The better potency of cinnamate **1** compared to that of piperine **2** also indicates that esters are better than amides.

The small molecules from natural and synthetic sources have been reported to be effective in inhibiting the induced expression of cell adhesion molecules on endothelial cells (*35*, *36*). For example, aspirin, mesalamine, and phenyl methimazole inhibit the TNF-α-induced expression of VCAM-1 at IC_{50} values of 6000, 16 000, and 500 μM, respectively (*37*−*39*). Similarly, diclofenac, *N*-acetylcysteine, and pyrrolidone dithiocarbamate are most effective at concentrations of 750, 100, and 1000 μM, respectively (*40*, *41*). In comparison, the IC_{50} value of novel compound **1** is ∼100 μM. This concentration is comparatively lower if one considers the above examples of lead molecules or drugs that are in clinical use. Therefore, these results indicate that our novel compound is potentially effective and, therefore, could be useful for further pharmaceutical studies.

Our kinetic studies (Figure 4) indicated that 3′,4′,5′-trimethoxycinnamate interferes with some earlier steps of the TNF-α signaling cascade. In the downstream of the TNF-α signaling cascade, a number of kinases, including IKKα and IKKβ, become activated which are necessary for IκB phosphorylation and NF-κB activation (*42*). Nuclear transcription factor NF-κB is involved in the regulation of the expression of cell adhesion molecules (*19*). Binding sites for NF-κB have been identified in the promoter regions of the genes for ICAM-1, VCAM-1, and E-selectin (*43*−*45*). Although the mechanism of inhibition of cell adhesion molecules by 3′,4′,5′-trimethoxycinnamate and its analogues is not investigated here, it is tempting to speculate that they may be interfering with the NF-κB activation pathway. In any event, the exact mechanism of action responsible for inhibition of cell adhesion molecules remains to be elucidated. Importantly, our results of identifying and characterizing 3′,4′,5′-trimethoxycinnamate and its analogues have implications in discovering therapeutically valuable lead molecules against various inflammatory diseases.

SUPPORTING INFORMATION AVAILABLE

Complete spectra of novel compounds **4** and **10**. This material is available free of charge via the Internet at http://pubs.acs.org.

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