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ARTICLE

Isolation and Characterization of *N*-Feruloyltyramine as the P-Selectin Expression Suppressor from Garlic (*Allium sativum*)

JAE B. PARK*

Diet, Genomics, and Immunology Laboratory, Building 307C, Room 131, BHNRC, ARS, USDA, Beltsville, Maryland 20705

Garlic (*Allium sativum*) is a medicinal and culinary plant reported to have several positive health effects on cardiovascular diseases, particularly via suppressing platelet activation. Therefore, active compounds inhibiting platelet activation were isolated from garlic extract using a P-selectin expression suppressing activity-guided fractionation technique. Garlic cloves were extracted with methanol, sequentially partitioned using ethyl acetate, and *n*-butanol. The ethyl acetate portion was fractionated using silica gel chromatography. The fraction with highest P-selectin expression suppressing activity was further purified using HPLC, and the compounds in the fraction were analyzed using MS, MS/MS, and NMR spectroscopic methods. Using NMR spectroscopy, the compound with highest suppressing activity was confirmed as *N*-feruloyltyramine. At the concentration of 0.05 μM, *N*-feruloyltyramine was able to suppress P-selectin expression on platelets by 31% ($P < 0.016$). Since COX enzymes are deeply involved in the regulation of P-selectin expression on platelets, potential effects of *N*-feruloyltyramine on COX enzymes were investigated. As expected at the concentration of 0.05 μM, *N*-feruloyltyramine was found to be a very potent compound able to inhibit COX-I and -II enzymes by 43% ($P < 0.012$) and 33% ($P < 0.014$), respectively. *N*-Feruloyltyramine is likely to inhibit COX enzymes, thereby suppressing P-selectin expression on platelets.

KEYWORDS: ***Allium sativum*** **(garlic); *N*-feruloyltyramine; COX inhibition; platelet activation; P-selectin**

INTRODUCTION

Allium sativum (garlic) is a well-known medicinal plant belonging to the family Liliaceae, commonly used as a culinary spice worldwide (*1*, *2*). The genus *Allium* consists of more than 900 species including *A. cepa* (onion), *A. fistulosum* (Chinese chive), *A. porrum* (leek) and *A. tuberosum* (green onion). The plants belonging to this genus contain numerous phytochemicals such as phenolics, terpenoids, alkaloids and sulfur-containing compounds (*3*−*5*). Traditionally, garlic is believed to have antithrombotic, antihypertensive, anticholesterol, carminative, antioxidant, antimicrobial, and antimutagenic activities (*6*−*8*). Several recent studies suggest that garlic and its products have beneficial health effects on atherosclerotic cardiovascular disease, of which platelet activation is critically involved in the progression (*9*, *10*). Platelet activation and its related events are involved in the pathophysiology of several cardiovascular diseases such as atherosclerosis, angina, acute myocardial infarction, and ischemic cerebral stroke (*11*−*15*). Platelet activation is often triggered by the exposure to ADP (adenosine diphosphate), collagen, thrombin, thromboxane A2 (TXA2), and/or arachidonic acid derivatives. Upon the activation, P-selectin that is stored in the alpha-granules of platelets is trans-located to the cell surface, facilitating adhesion to leukocytes and/or endothelium, and eliciting the cells to produce numerous biomolecules such as cytokines, tissue factors, and pro-coagulants (*16*−*20*). Therefore, compounds able to suppress P-selectin expression have been a great research interest in the perspective of the pathogenesis of coronary artery and others diseases (*16*, *17*).

Garlic and its products have been long studied for their potential health effects, and numerous chemicals from garlic have been identified, isolated, and characterized as beneficial to several human diseases (*4*−*7*). Recent studies indicated that garlic may have important positive health effects on cardiovascular diseases more than any others (*5*−*7*). However, not many studies have been conducted to identify or characterize compounds from garlic using specific biomarkers related to cardiovascular diseases. In our laboratory, compounds suppressing P-selectin expression have been evaluated from plant sources for the last ten years. In this study, garlic extracts were prepared from the edible parts of garlic cloves, and biologically active compounds were isolated and characterized from the extract using a P-selectin expression suppressing activity-guided fractionation technique. Among the compounds isolated from garlic extract, the one with highest P-selectin suppressing activity was identified, using MS, MS/MS, NMR spectroscopic methods, as *N*-feruloyltyramine. Also, the effect of compounds isolated from garlic on COX enzymes (I and II) was investigated because COX enzymes are critically involved in P-selectin expression on platelets.

MATERIALS AND METHODS

Materials. COX-I and -II enzymes and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Fluorescein isothiocyanate (FITC)-conjugated rat antimouse CD62p monoclonal antibody

*Tel: 301-504-8365 Fax: 301-504-9456 E-mail: jae.park@ars.usda.gov.

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and the isotype control were purchased from BD Biosciences (Camarillo, CA). Garlic was obtained from Christopher Ranch Farm (Gilroy, CA). *N*-Feruloyltyramine, *N*-caffeoyltyramine, *N*-caffeoylnorephedrine and *N*-cinnamoyltyramine were synthesized and purified (more than 96% purity) as described previously (*21*−*23*).

Extraction and Fractionation. Clean and peeled garlic cloves (500 g) were extracted three times with MeOH (2 L) at room temperature (2 days each). The MeOH extract was evaporated in vacuo to yield a dark brown residue, which was suspended in H_2O (0.5 L) and then partitioned sequentially using EtOAc and *n*-BuOH (3 × 0.5 L) as solvent. The EtOAc fraction was passed through a silica gel column (120 × 10 cm) and eluted using solvent mixtures of *n*-hexane and EtOAc with increasing polarity as eluents. The fractions were individually heat-dried (95 °C) to a brown residue and dissolved in 10% ethanol (15 mL).

HPLC Purification. *N*-Feruloyltyramine from standard and garlic extract samples was prepared in 10% ethanol. A 150 mm × 2.1 mm i.d., 4 μm, Nova-Pak C18 (Waters, Milford, MA) was used as the stationary phase to analyze *N*-feruloyltyramine standard and garlic samples. The samples were separated using a gradient condition (buffer A (50 mM NaH_2PO_4, pH 4.3) for 0−5 min, a linear change from buffer A to buffer B (Buffer A containing 40% methanol) for 5−25 min, and buffer B for 10 min at the flow rate of 1 mL/min. The samples were injected by an autosampler into Alliance 2690 HPLC system (Waters, Milford, MA), and detection was carried out a by CoulArray electrochemical detector with four electrode channels (ESA, Chelmsford, MA). The amount of *N*-feruloyltyramine (more than 93% purity) in the samples was quantified by CoulArray electrochemical detector software (v.1.0).

LC−MS and LC−MS/MS analyses. The garlic and *N*-feruloyltyramine standard samples were prepared for LC−MS and LC−MS/MS analyses (*22*, *23*). LC−MS was performed with an Agilent 1100 HPLC system coupled with LCQ class ion-trap mass spectrometer using electrospray ionization (ESI) in positive ion mode. A Supelco C18 (250 mm × 3.0 mm i.d., 3 μm) reversed-phase column operated at 25 °C at a flow rate of 0.25 mL/min. The mobile phases were water and acetonitrile containing 0.1% formic acid as described previously (*23*). For the verification of the MS peak as *N*-feruloyltyramine, LC−MS/MS was also performed to monitor the fragments of *N*-feruloyltyramine from *m*/*z* 80 to 400, with the following conditions: sheath gas flow rate, 70 (arbitrary units); auxiliary gas flow rate, 10 (arbitrary units); spray voltage, 4.50 kV; heated capillary temperature, 220 °C; capillary voltage, −4.0 V; tube lens offset, 25 V. The collision energy for the LC−MS/MS was set at 30%.

NMR Analysis. For NMR experiments, the sample was prepared by dissolving *N*-feruloyltyramine from garlic (25 mg) in d_6-DMSO (0.75 mL). ^1H, ^{13}C, COSY, HSQC and HMBC spectra were acquired at ambient temperature on the JEOL BCX-400 NMR spectrometer operating 400 MHz for ^1H and 100 MHz for ^{13}C. Chemical shifts were referenced to DMSO (2.50 ppm for ^1H, 39.5 ppm for ^{13}C).

Blood Samples. Swiss Webster male mice 3−4 weeks old were purchased from Charles River (Wilmington, MA). Mice were placed in standard cages and housed in the environmentally controlled Beltsville Human Nutrition Research Center Animal Facility. The protocol of animal study was approved by Beltsville area animal care and use committee (BAACUC). On arrival, mice were fed AIN-76A purified diet that provides the recommended allowance of all nutrients required for maintaining optimal health, but lacking *N*-feruloyltyramine and its analogues (*N*-caffeoyltyramine, *N*-caffeoylnorephedrine and *N*-cinnamoyltyramine), which was confirmed by HPLC analyses. Whole blood was collected from mice, via tail bleeding technique, and used for measuring P-selectin expression on platelets.

Measurement of P-Selectin Expression. Blood was collected from mice in siliconized microfuge tubes containing 15% EDTA. The modified Tyrodes buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, and 0.35% (w/v) bovine serum albumin, pH 7.0) was added to bring the sample volume to 100 μL. From the blood samples, aliquots were placed in 12 × 75 polypropylene tubes along with the appropriate antibody, and the modified Tyrodes buffer in a final volume of 200 μL. The purified extract, *N*-feruloyltyramine and analogues were dissolved in 10% ethanol, and added to diluted blood samples, where the final ethanol volume never exceeded 1% (v/v) in both control and test tubes. Samples were analyzed for P-selectin (CD62p) expression on platelets within three hours of the collection by flow cytometry (*24*, *25*). Data were acquired for 30,000 platelets, and the extent of expression of CD62p was determined as the measure of platelet activation (*25*).

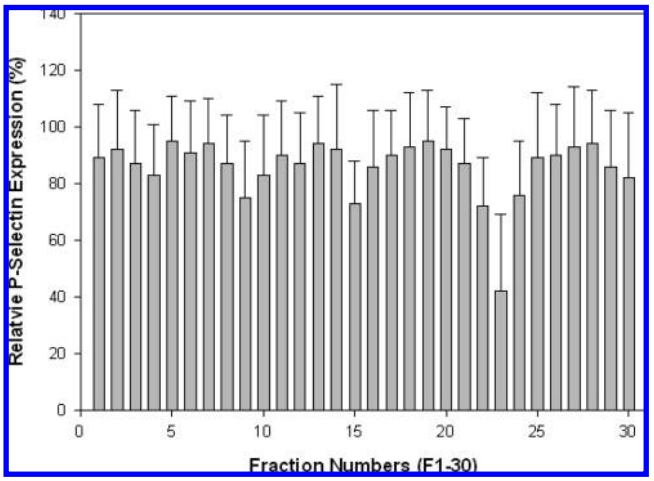

Figure 1. Extraction and fractionation of compounds suppressing P-selectin expression from *Allium sativum*. Garlic extract was fractionated using a silica gel column, and thirty fractions (F1−30) were collected and assayed for P-selectin suppressing activity. The highest suppressing activity was detected in fraction F23, and some moderate suppressing activity was also detected in fractions F9 and F15.

Cyclooxygenase (COX) I and II Inhibition Assay. COX-I and -II activities were measured in a 96-well plate using a chemiluminescent COX kit (Assay Designs Inc., Ann Arbor, MI). Briefly, 50 μL of Tris-phenol buffer (100 μM Tris, 0.5 μM phenol buffer, pH = 7.3) was added into the wells. 50 μL of hematin solution (hematin was dissolved in DMSO at 0.380 mg/mL, and diluted 5,000-fold with 100 mM phosphate buffer, pH = 7.5) was added and 50 μL of COX-I (700 units) or COX-II (700 units) was added into the wells. The samples were incubated at room temperature for 5 min. After the incubation, *N*-feruloyltyramine, its analogues, or COX inhibitors were added, and the samples were incubated at room temperature (in the dark) for 10 min. COX activity was measured using a luminometer, by injecting 50 μL of chemiluminescent COX substrate (4 °C) and arachidonic acid, respectively. Relative light units (RLU) output was measured to determine COX activity.

Statistical Analysis. Treatment effects on the parameters measured were compared by analyzing the means for differences using either ANOVA or ANOVA. Differences were considered to be significant when $p < 0.05$. Data points represent the mean ± SD of three or more samples.

RESULTS AND DISCUSSION

Extraction and Fractionation of P-Selectin Expression Suppressing Compounds. Since garlic clove is a major part of garlic for human consumption, edible parts of cloves were extracted with MeOH in this experiment. The MeOH extract was evaporated, partitioned and passed through a silica gel column as described in Materials and Methods. From the silica column, thirty fractions (F1−30) were collected, and heat-dried individually to a dark brown residue, which was then dissolved in 10% ethanol for P-selectin assay. As shown in **Figure 1**, the highest suppressing activity was detected in fraction F23, although some fractions (F9 and F15) were also found to have moderate suppressing activity.

HPLC Purification of Compounds Suppressing P-Selectin Expression. Fraction F23 was heat-dried, and the residue was prepared in 10% ethanol, injected into HPLC, and separated using a gradient condition described in Materials and Methods. Thirty-five factions were collected at one min intervals. A dominant peak was detected at the retention time of 20.7 min (**Figure 2A**), with the greatest P-selectin expression suppressing activity (**Figure 2B**).

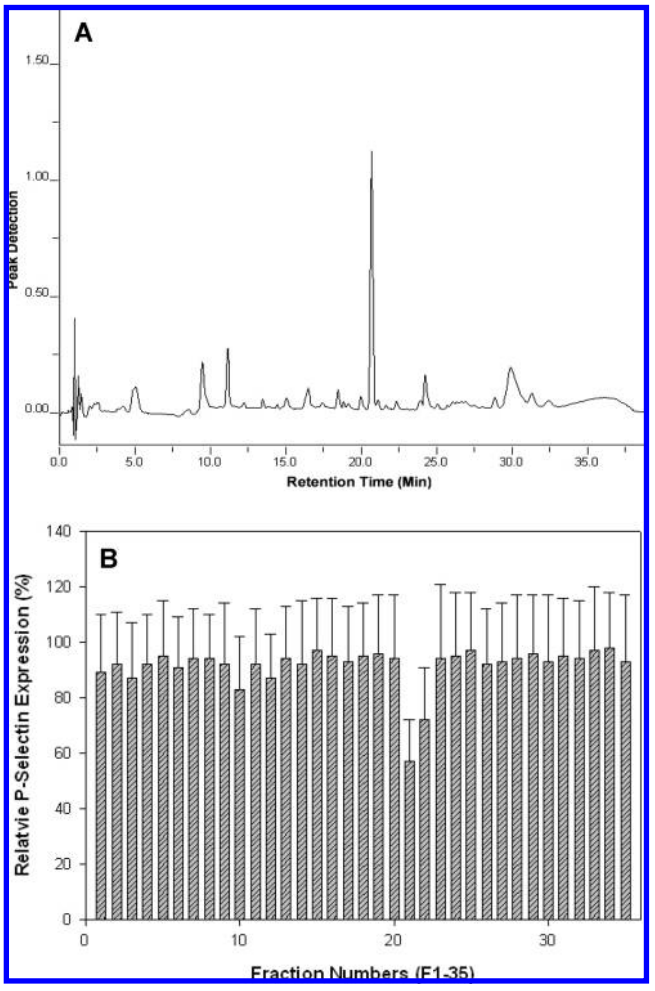

Figure 2. HPLC chromatography and P-selectin expression suppressing activity.

(**A**) The HPLC chromatogram of fraction F23. A dominant peak was detected at the retention time of 21 min. (**B**) The HPLC fractions of F23 were collected and assayed for P-selectin expression suppressing activity.

LC−MS and LC−MS/MS Analyses. The peak with highest P-selectin expression suppressing activity was purified by HPLC, and the sample was prepared for LC−MS and LC−MS/MS analyses (23). The LC−MS chromatogram showed a major peak, and the major signal from the peak was at *m*/*z* 313.5, which is probably from *N*-feruloyltyramine (**Figure 3A**). Therefore, LC−MS was performed using synthesized *N*-feruloyltyramine and it was shown that the both major peaks from the HPLC-purified garlic and standard *N*-feruloyltyramine samples were identical at *m*/*z* 313.5 (data not shown). In order to verify the major signal as *N*-feruloyltyramine, LC−MS/MS experiments were performed using the product of the major signal in LC−MS. As shown in **Figure 3B**, LC−MS/MS spectrum showed three peaks (*m*/*z* 122, 137 and 178), which could be ions of 4-ethylphenol, tyramine and ferulic aldehyde, respectively. All three peaks are daughter ions which can be derived from *N*-feruloyltyramine, indicating that the compound in the peak of the LC−MS chromatogram is likely to be *N*-feruloyltyramine.

NMR Analyses. To unambiguously confirm the identity of *N*-feruloyltyramine, NMR analyses were performed. The NMR data were the following: ^{1}H NMR (d_6-DMSO, 400 MHz) δ 7.50 (1H, d, J = 15.7 Hz, H-7), 7.06 (H, d, J = 8.4 Hz, H-13), 7.01 (1H, dd, J = 8.2, 1.5 Hz, H-5), 6.48 (1H, d, J = 15.7 Hz, H-8), 6.83 (1H, d, J = 1.5 Hz, H-4), 6.77 (1H, d, J = 8.4 Hz, H-14), 7.08 (1H, d, J = 8.2 Hz, H-1), 3.82 (1H, s, H-16), 3.51 (1H, t, J = 7.5 Hz, H-10), 2.78 (1H, t, J = 7.5 Hz, H-11); ^{13}C NMR (d_6-DMSO, 100 MHz) δ 167.9 (C-9), 155.5 (C-15), 148.5 (C-3), 147.9 (C-2), 140.9 (C-7), 130.0 (C-12), 129.5 (C-13), 127.0 (C-6), 122.0 (C-5), 117.6 (C-8), 115.3 (C-4), 115.1 (C-14), 110.3 (C-1), 55.1 (C-16), 41.3 (C-10), 34.5 (C-11). Based on NMR data, the structure of the HPLC purified compound with P-selectin expression suppressing activity was determined as being 3-(4-hydroxy-3-methoxy-phenyl)-*N*-[2-(4-hydroxy-phenyl)-ethyl]-acrylamide (*N*-feruloyltyramine) (**Figure 4**).

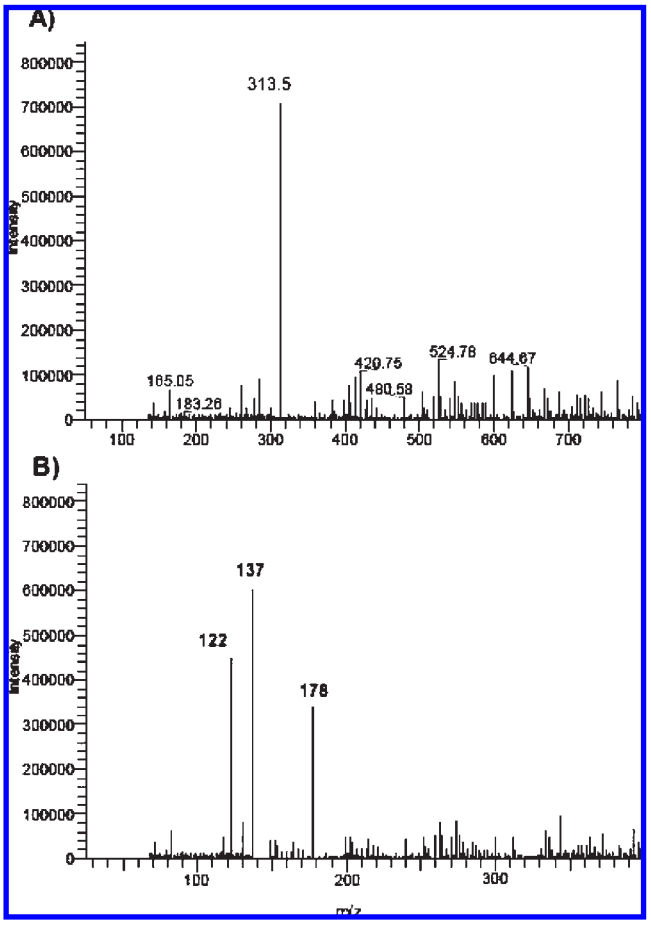

Figure 3. LC−MS and LC−MS/MS analyses.

(**A**) LC−MS was performed, and a major peak was detected at *m*/*z* 313.5. (**B**) MS/MS was performed, and three daughter ions were detected at *m*/*z* 122, 137, and 178.

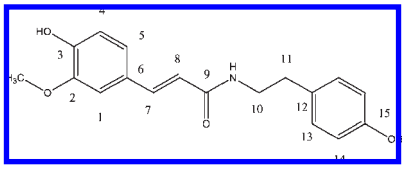

Figure 4. The structure of *N*-feruloyltyramine.

Inhibitory Effect of *N*-Feruloyltyramine on COX-I and -II. Cyclooxygenases (COX-I and -II) are expressed in a variety of cell types involved in prostaglandin homeostasis (*26*). In particular, COX-I inhibition is known to prevent platelet activation via numerous mechanisms, including the suppressing of P-selectin expression. Therefore, the effects of *N*-feruloyltyramine on COX-I were investigated to elucidate the mechanism underlining the suppression of P-selectin expression. As shown in **Figure 5A**, *N*-feruloyltyramine was able to inhibit COX-I enzyme by 43% (P < 0.013) at a concentration of 0.05 μM comparable to the

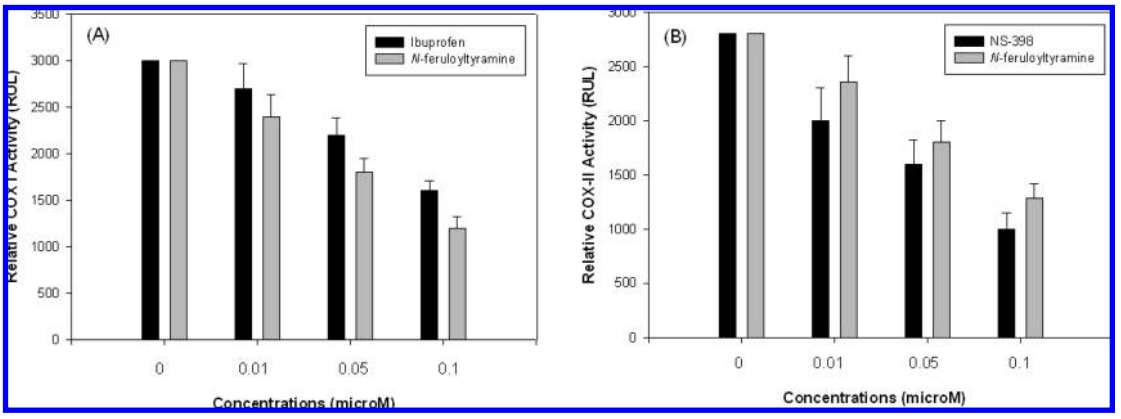

Figure 5. Effects of *N*-feruloyltyramine on COX-I and -II.

N-Feruloyltyramine and COX inhibitors (0, 0.01, 0.5, and 0.1 μM) were added to the samples, and the reaction mixtures were incubated for 10 min. Following the incubation, COX-I (**A**) and -II (**B**) activities were measured according to the kit's protocol using a luminometer.

well-known COX-I inhibitor ibuprofen. Although COX-I inhibition is primarily involved in suppressing P-selectin expression on platelet, the effects of *N*-feruloyltyramine on COX-II were also investigated because COX-II is involved in many related physiological processes. Similar to COX-I inhibition, *N*-feruloyltyramine was able to inhibit COX-II enzyme by 33% ($P < 0.015$) at a concentration of 0.05 μM, which was marginally less potent than the COX-II specific inhibitor NS-398 (**Figure 5B**). The data suggest that *N*-feruloyltyramine may be a potent compound able to inhibit both COX-I and -II enzymes.

Effects of *N*-Feruloyltyramine and Its Analogues on P-Selectin Expression. P-Selectin (CD62P) is a trans-membrane glycoprotein, commonly used as a reliable biomarker for determining platelet activation (*17*−*20*). The expression of the protein is reported to be greatly modulated by COX enzymes (*26*). This and previous studies indicate that *N*-feruloyltyramine and its analogues are likely to have COX inhibitory activity (*25*). Therefore, the effects of three compounds (*N*-feruloyltyramine, *N*-caffeoyltyramine and *N*-caffeoylnorephedrine) on P-selectin expression on platelets were determined as a means to assess the inhibition of platelet activation. As shown in **Figure 6**, all three *N*-feruloyltyramine analogues at the concentration of 0.05 μM were able to suppress P-selectin expression on platelets by 31% ($P < 0.010$), 39% ($P < 0.013$) and 30% ($P < 0.015$), respectively. In addition, all three compounds were found to be very potent inhibitors of COX-I (data not shown). Accordingly, their P-selectin suppressing activity was positively correlated with their COX inhibitory activity, suggesting that the COX-I inhibition may be a significant contributing factor to P-selectin expression suppression.

In the United States, cardiovascular diseases are still major causes of human mortality and morbidity (*27*, *28*). Therefore, from both nutritional and medical perspectives, the prevention of cardiovascular diseases has been a major research focus for many years. Atherosclerosis is one of the well-known cardiovascular diseases where inflammatory processes are aggravated following the blockage of arteries. During this process, excessive platelet activation is a significant contributor to the pathophysiological progress of atherosclerosis. In fact, excessive platelet activation is also complicated in other cardiovascular diseases such as angina, acute myocardial infarction, and ischemic cerebral stroke (*29*). Platelet activation can be greatly attenuated by compounds inhibiting COX enzymes and/or producing cAMP (*30*). In the process of platelet activation, P-selectin expression is induced and serves as a reliable biomarker for measuring platelet activation. P-Selectin is a 140 kDa type-1 trans-membrane glycoprotein belonging to the selectin family of cell adhesion membrane

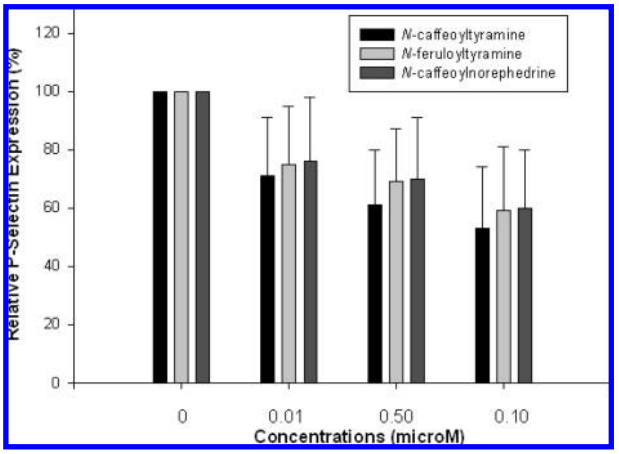

Figure 6. Suppression of P-selectin expression on platelets using *N*-feruloyltyramine and its analogues. Platelets were incubated with *N*-feruloyltyramine, *N*-caffeoyltyramine and *N*-caffeoylnorephedrine for 30 min. P-Selectin expression was determined as described in Materials and Methods. Data points represent the mean ± SD of five samples.

proteins (*16*−*20*). The P-selectin membrane protein is involved in platelet−leukocyte interactions and platelet−endothelium interactions via binding to P-selectin ligand (PSGL-1) on leukocytes and endothelium (*19*). Recently, several studies suggested that the risk factors of inflammatory cardiovascular diseases such as arthrosclerosis may be attenuated by healthy diets containing a variety of fruits and vegetables (*31*, *32*). An accumulating body of evidence also suggests that garlic (*Allium sativum*) may contribute to the prevention of arthrosclerosis by reducing critical inflammatory processes (*9*, *10*). However, compounds with beneficial effects on cardiovascular diseases have not been previously identified from garlic using P-selectin as a biomarker. Therefore, in this study, compounds were isolated from garlic using a P-selectin expression suppressing activity-guided fractionation technique. In this study, garlic cloves were used for preparing the extract in order to provide a relevant nutritional perspective. During the study, five different garlic extracts were prepared and *N*-feruloyltyramine was identified as an active compound by HPLC. For the period of the separation, the amount of *N*-feruloyltyramine in the samples was also quantified by an electrochemical detector using synthesized *N*-feruloyltyramine as a standard. The data indicated that the amounts of *N*-feruloyltyramine in garlic extracts were in the range of 5−35 mg per kg of *A. sativum*. Notably, dehydrated garlic samples contained less

N-feruloyltyramine than other garlic samples (data not shown). However, it is not known how much dehydration can reduce the amount of *N*-feruloyltyramine in garlic. Besides *N*-feruloyltyramine, other compounds (e.g., ferulic acid, *N*-caffeoyltyramine) were also isolated and found to have COX-inhibitory activity. The presence of ferulic acid is not surprising because the acid is a precursor for *N*-feruloyltyramine and related chemicals found in garlic. However, it is quite interesting that *N*-caffeoyltyramine can be found in the clove of garlic, which can be an immediate precursor for producing *N*-feruloyltyramine via the O-methylation of the 3-hydroxyl group of phenylpropenic acid moiety. However, the amount of *N*-caffeoyltyramine was approximately 10 times less than that of *N*-feruloyltyramine, which may explain the reason why *N*-feruloyltyramine was isolated as a dominant garlic compound able to suppress P-selectin expression on platelets, even though *N*-caffeoyltyramine could inhibit P-selectin expression. During the study, the efficacy of *N*-feruloyltyramine and its analogues (e.g., *N*-caffeoyltyramine, *N*-caffeoylnorephedrine, *N*-cinnamoyltyramine) in inhibiting COX enzymes was investigated, and these compounds were found to be quite potent COX-I inhibitors except *N*-cinnamoyltyramine (data not shown). A main structural difference between *N*-cinnamoyltyramine and the rest is to have hydroxyl groups at the 3- and/or 4-positions of the phenylpropenoic acid moiety. This suggests that hydroxyl groups at the 3- and 4-positions of the phenylpropenoic acid may be critical in inhibiting COX-I. This assumption was further supported by our previous observation that *N*-coumaroyltyramine was better than *N*-cinnamoyltyramine, but not as good as *N*-caffeoyltyramine, in inhibiting COX enzymes (*25*).

In this study, *N*-feruloyltyramine was isolated from garlic extract and found to suppress P-selectin expression on platelets probably via inhibiting COX enzyme. Although COX inhibition is a powerful target for suppressing P-selectin expression, our previous and other studies suggested that additional mechanisms induced by compounds such as cAMP and arachidonic acid derivatives contribute to suppressing P-selectin expression on platelets (*25*, *33*). Currently, the activity of *N*-feruloyltyramine on cAMP and other biological molecules is under investigation. Therefore, our ongoing investigations may provide information about the use of natural plant-derived *N*-feruloyltyramine and its analogues as P-selectin expression suppressors with potentially better efficacy in preventing and/or treating cardiovascular and other related diseases in the perspective of nutrition.

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