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Impact of Alkyl Esters of Caffeic and Ferulic Acids on Tumor Cell Proliferation, Cyclooxygenase Enzyme, and Lipid Peroxidation

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The antioxidant ferulic and caffeic acid phenolics are ubiquitous in plants and abundant in fruits and vegetables. We have synthesized a series of ferulic and caffeic acid esters and tested for tumor cell proliferation, cyclooxygenase enzymes (COX-1 and -2) and lipid peroxidation inhibitory activities in vitro. In the tumor cell proliferation assay, some of these esters showed excellent growth inhibition of colon cancer cells. Among the phenolics esters assayed, compounds **10** (C_{12}-caffeate), **11** (C_{16}-caffeate), **21** (C_8-ferulate), and **23** (C_{12}-ferulate) showed strong growth inhibition with IC_{50} values of 16.55, 13.46, 18.67, and 7.57 μg/mL in a breast cancer cell line; 9.65, 7.45, 17.05, and 4.35 μg/ mL in a lung cancer cell line; 5.78, 3.5, 4.29, and 2.46 μg/mL in a colon cancer cell line; 12.04, 12.21, 14.63, and 8.09 μg/ mL in a central nervous system cancer cell line; and 8.62, 7.76, 11.0, and 5.37 in a gastric cancer cell line. In COX enzyme inhibitory assays, ferulic and caffeic acid esters significantly inhibited both COX-1 and COX-2 enzymes. Caffeates **5**−**10** (C_4−C_{12}), inhibited COX-1 enzyme between 50% and 90% and COX-2 enzyme by about 70%, whereas ferulates **15**−**21** (C_3−C_8) inhibited COX-1 and COX-2 enzymes by 85−95% 25 μg/mL. Long-chain caffeates **11**−**14** (C_{16}−C_{22}) and short-chain ferulates **15**−**20** (C_3−C_5) were the most active in lipid peroxidation inhibition and showed 60−70% activity at 5 μg/mL concentration.

KEYWORDS: Alkyl esters of caffeic and ferulic acids; antitumor; antiinflammatory; antioxidant

INTRODUCTION

The antioxidant phenolics—ferulic, caffeic, sinapic, chlorogenic, and *p*-coumaric acids and their conjugates—are found in whole grain, coffee beverages, sunflower, blueberries, apples, spinach, grapes, sweet cherries, and other fruits and vegetables (*1*, *2*). They usually exist as glycosides or bound to proteins and cell wall polymers. Epidemiological studies suggest a link between the consumption of whole grain products containing ferulic acid or its derivatives and prevention of coronary heart diseases and certain forms of cancers (*3*). These compounds are well-known as antioxidants and prevent oxidative damage of DNA by several mechanisms. In addition, some of these phenolics are also known to exhibit antiinflammatory (*4*), antiproliferative (*5*), antiviral (*6*), and immunoprotective properties (*7*). The suggested mechanism of phenolic compounds as anticancer agents involves the induction of detoxification systems, specifically the phase II conjugation reactions (*8*). Although alkyl esters of ferulic and caffeic acids are widely distributed in food products, their beneficial biological activities are not fully explored.

Cancer incidence and morbidity are steadily increasing in several parts of the world even though there is a decline in certain cancers in the United States (*9*). It is the result of complex interplay between genetic susceptibility and environmental factors. The phytochemicals, carotenoids, chlorophyll, flavonoids, isothiocyanate, and polyphenolics present in fruits, vegetables and whole grains are considered to modulate the cancer initiation step.

Cyclooxygenase (COX) enzymes catalyze the key step in the inflammatory process, conversion of arachidonic acid to prostaglandins (PGH_2). Prostaglandins play critical roles in numerous biologic processes, including the regulation of immune function, kidney development, reproductive biology, and gastrointestinal integrity. Also, COX-2 is overexpressed in several types of cancers, and hence it is one of the targets for the development of anticancer drugs (*10*). The toxic intermediates such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (−OH•) formed during the conversion of respiratory oxygen to H_2O are highly reactive and induce lipid peroxidation, cross-linking of the proteins, DNA scissions, and decreased mitochondrial function (*11*). It is also reported that the tumor

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promoters recruit the inflammatory cells to stimulate the reactive oxygen species and promote cancer development (*12*, *13*). Therefore, the antioxidants and antiinflammatory agents in food play an important role in scavenging free radicals and limit the initiation and progression of tumors. In this paper, we report the synthesis of alkyl esters of ferulic and caffeic acids and their health beneficial biological activities.

MATERIALS AND METHODS

General Experimental. The NMR (^{1}H and ^{13}C) experiments were carried out by using Varian VXR 500 and 300 instruments. The chemical shifts were measured in $CDCl_3$ or acetone-d_6 and are expressed in δ (parts per million, ppm). ACS-grade solvents were used for purification. The COX-1 enzyme was prepared from ram seminal vesicles purchased from Oxford Biomedical Research, Inc. (Oxford, MI). The COX-2 enzyme was prepared from insect cells cloned with human PGHS-2 enzyme. Aspirin, ibuprofen, naproxen, and Vioxx were used as positive controls in COX enzyme inhibitory assays. Fetal bovine serum (FBS) and Roswell Park Memorial Institute-1640 (RPMI-1640) medium were obtained from Gibco BRL (Grand Island, NY). Human tumor cell lines MCF-7 (breast), SF-268 (central nervous system, CNS) and NCI-H460 (lung) were purchased from the National Cancer Institute (NCI, Bethesda, MD). HCT-116 (colon) and AGS (gastric) cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD). Phospholipid 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocoline (SLPC) was purchased from Avanti Polar Lipids (Alabaster, AL). Fluorescent probe 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl]phenylpropionic acid was purchased from Molecular Probes (Eugene, OR). Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tert*-butylhydroquinone (TBHQ), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], ferulic acid, caffeic acid, and corresponding alkanols were purchased from Sigma−Aldrich Chemical Co. (St. Louis, MO). Concentrations are expressed in micrograms per milliliter.

Tumor Cell Proliferation Inhibitory Assay. MCF-7 (breast), SF-268 (CNS), NCI-H460 (lung), HCT-116 (colon), and AGS (gastric) human tumor cells were cultured in RPMI-1640 medium containing penicillin−streptomycin (10 units/mL for penicillin and 10 μg/mL for streptomycin) and 10% FBS. The cells were grown in a humidified incubator (37 °C, 5% CO_2), counted, and plated into 96-well plates. The number of cells for each cell line varied according to their doubling time. The samples were dissolved in dimethyl sulfoxide (DMSO) and further diluted with RPMI medium to obtain a stock solution that gave the desired concentration of 20 μg/mL and a DMSO concentration of 0.2%. Lower concentrations of test samples were prepared by serial dilution of the stock solutions with RPMI medium. After 24 h of incubation, test samples (100 μL) were added to the wells containing the appropriate tumor cells and incubated for an additional 48 h. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (5 mg) was dissolved in PBS (phosphate-buffered saline; 1 mL) and aliquots of 25 μL were added to each well. The cell viability was determined according to the published procedure (*14*). The samples were assayed in triplicate, and two or three independent experiments were carried out to calculate either percent inhibition or IC_{50} values.

Cyclooxygenase Inhibitory Assay. The COX enzyme inhibitory assay was performed in a micro chamber at 37° C by monitoring the initial rate of O_2 uptake with an oxygen electrode (Instech Laboratories, Plymouth Meeting, PA) attached to a biological oxygen monitor (Yellow Springs Instrument, Inc., Yellow Springs, OH) (*14*). The enzyme was diluted with Tris buffer (pH 7.0) to give a final concentration of 1.5 mg of protein/mL. The test compounds and positive controls were dissolved in DMSO. An aliquot of 10 μL of DMSO or test compounds or standards in DMSO was added to the reaction chamber containing 0.6 mL of 0.1 M Tris buffer (pH 7), 1 mM phenol, and hemoglobin (17 μg). COX-1 or -2 enzyme (10 μL) was added to the chamber and incubated for 3 min. The reaction was initiated by the addition of arachidonic acid (10 μL of 1 mg/mL solution). Instantaneous inhibition was measured by using Quick Log Data acquisition and control computer software (Strawberry Tree Inc., Sunnyvale, CA). The percent inhibition was calculated with respect to DMSO control. Each sample was assayed two times and the standard deviation was calculated for $n = 2$.

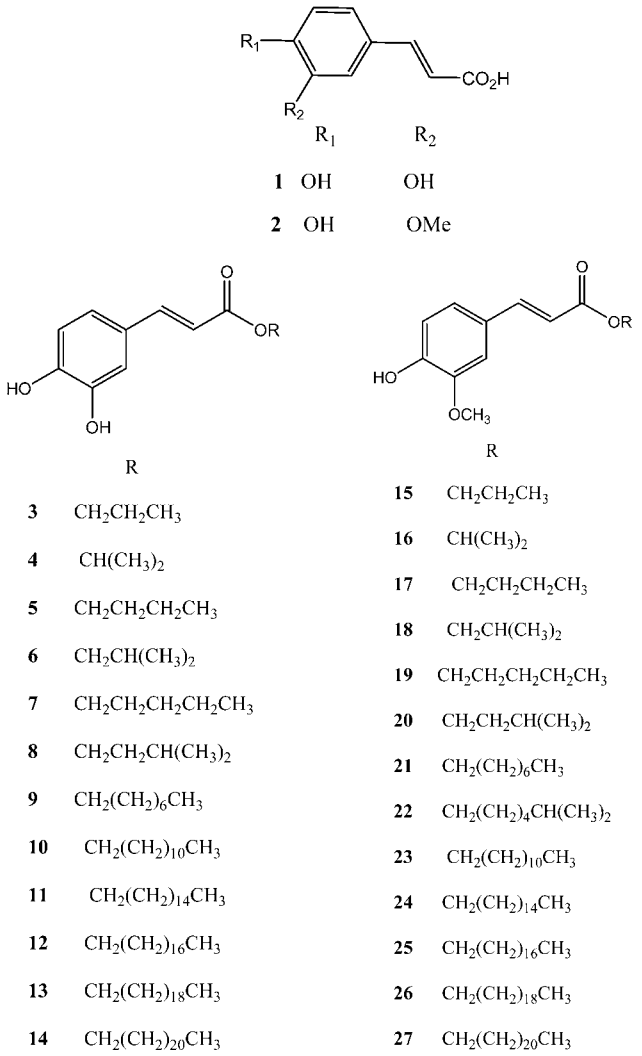

Figure 1. Structures of compounds **1**–**27**.

Lipid Peroxidation Inhibitory Assay. Phospholipid 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocoline (SLPC) in $CHCl_3$ (10 mg/mL) and fluorescence probe 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl]phenylpropionic acid (DPH-PA) in *N*,*N*-dimethylformamide (DMF) (1 mg/mL) were mixed and evaporated under reduced pressure to yield a residue. It was then freeze−thawed in 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer and homogenized with an extruder (Avestin Inc., Ottawa, ON, Canada) to yield large unilamellar vesicles (LUVs). The lipid peroxidation inhibitory assay was conducted by using these LUVs according to the previously published procedure from our laboratory (*15*).

Synthesis of Caffeic Acid Esters. Compounds **7**−**14** were synthesized by reacting caffeic acid and corresponding alkanols with thionyl chloride ($SOCl_2$) as the solvent (**Figure 1**). First, caffeic acid (1 mM) and thionyl chloride ($SOCl_2$, 1 mL) were stirred at 90 °C until the reaction mixture became a homogeneous solution (*16*). To this solution, the desired alcohol (1 mM) was added dropwise, and the reaction was allowed to continue for an additional 4−24 h. The longer chain alcohols required a longer reaction time to yield products. The reaction was monitored by thin-layer chromatography (TLC). After completion of the reaction, $SOCl_2$ was evaporated under reduced pressure. The resulting residue was purified by silica gel column with $CHCl_3$/MeOH (9:1 and 8:2 v/v) as the mobile phase. Ten fractions (15 mL each) were collected. Similar fractions were combined on the basis of TLC, and solvent was evaporated. Fractions eluted with $CHCl_3$/MeOH (8:2 v/v) yielded desired alkyl esters and were further crystallized to yield

pure compounds. The caffeic acid esters **3**−**6** were prepared by Fischer esterification as described below for ferulate esters (**Figure 1**) (*17*). The structures of caffeic esters were confirmed by ^{1}H and ^{13}C NMR spectral studies (*18*−*20*).

Synthesis of Ferulic Acid Esters. Ferulic acids esters (**15**−**27**) were synthesized according to the reported procedure (**Figure 1**) (*17*). The ferulic acid (1 mM) and corresponding alcohols (1 mM) were stirred with dry toluene (2 mL). The catalyst, *p*-toluenesulfonic acid (catalytic amounts), was added to the reaction mixture,which was stirred for 4 h at 120 °C. It was then extracted with saturated $NaHCO_3$ solution and the organic layer was dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure, and the resulting syrupy mass was purified on a silica gel column with *n*-hexane/EtOAc (9:1 and 8:2 v/v) as the mobile phase. Fifteen fractions (15 mL each) were collected. Evaporation of fractions eluted with hexane/EtOAc (8:2 v/v) gave pure compounds. The structures of ferulic acid esters were confirmed by ^{1}H and ^{13}C NMR spectral experiments (*21*, *22*).

***n*-Propyl Caffeate (3).** ^{1}H NMR (acetone-d_6) δ 8.26 (2H, s, OH), 7.53 (1H, d, J = 16.2 Hz), 7.15 (1H, d, J = 2.1 Hz), 7.04 (1H, dd, J = 8.1, 2.1 Hz), 6.86 (1H, d, J = 8.1 Hz), 6.28 (1H, d, J = 16.2 Hz), 4.09 (2H, t, J = 6.6 Hz), 1.67 (2H, m), 0.95 (3H, t, J = 7.5 Hz). ^{13}C NMR (acetone-d_6) δ 167.2, 148.4, 146.1, 145.3, 127.5, 122.3, 116.2, 115.6, 115.0, 65.9, 22.6, 10.5.

Isobutyl Caffeate (6). ^{1}H NMR (acetone-d_6) δ 8.30 (2H, s, OH), 7.54 (1H, d, J = 16.2 Hz), 7.16 (1H, d, J = 1.8 Hz), 7.05 (1H, dd, J = 8.1, 1.8 Hz), 6.86 (1H, d, J = 8.1 Hz), 6.29 (1H, d, J = 16.2 Hz), 3.92 (2H, d, J = 6.6 Hz), 1.96 (2H, m), 0.95 (6H, d, J = 6.9 Hz). ^{13}C NMR (acetone-d_6) δ 167.2, 148.4, 146.0, 145.3, 127.4, 122.3, 116.1, 115.5, 115.0, 70.4, 28.4, 19.1.

Isopentyl Caffeate (8). ^{1}H NMR (acetone-d_6) δ 8.30 (2H, s, OH), 7.50 (1H, d, J = 15.9 Hz), 7.15 (1H, d, J = 2.1 Hz), 7.04 (1H, ddd, J = 8.1, 2.1, 0.3 Hz), 6.86 (1H, d, J = 8.1 Hz), 6.27 (1H, d, J = 15.9 Hz), 4.17 (2H, t, J = 6.9 Hz), 1.65 (1H, m), 1.58 (2H, m), 0.93 (6H, d, J = 6.6 Hz). ^{13}C NMR (acetone-d_6) δ 167.2, 148.5, 146.1, 145.3, 127.4, 122.3, 116.1, 115.5, 115.0, 62.8, 38.1, 25.6, 22.5.

Hexadecyl Caffeate (11). ^{1}H NMR (acetone-d_6) δ 8.31 (2H, br s, OH), 7.52 (1H, d, J = 16.2 Hz), 7.15 (1H, d, J = 2.1 Hz), 7.03 (1H, dd, J = 8.1, 2.1 Hz), 6.85 (1H, d, J = 8.1 Hz), 6.27 (1H, d, J = 16.2 Hz), 4.13 (2H, t, J = 6.6 Hz), 1.67 (2H, m), 1.27 (26H, br s), 0.86 (3H, t, J = 6.9 Hz). ^{13}C NMR (acetone-d_6) δ 167.3, 148.5, 146.1, 145.3, 127.5, 122.3, 116.2, 115.6, 115.0, 64.5, 32.5, 28.9, 26.5, 23.2, 14.2.

Octadecyl Caffeate (12). ^{1}H NMR (acetone-d_6) δ 8.31 (2H, br s, OH), 7.52 (1H, d, J = 16.2 Hz), 7.15 (1H, d, J = 2.1 Hz), 7.03 (1H, dd, J = 8.1, 2.1 Hz), 6.85 (1H, d, J = 8.1 Hz), 6.27 (1H, d, J = 16.2 Hz), 4.13 (2H, t, J = 6.6 Hz), 1.67 (2H, m), 1.27 (30H, br s), 0.86 (3H, t, J = 6.9 Hz). ^{13}C NMR (acetone-d_6) δ 167.3, 148.6, 146.2, 145.3, 127.4, 122.3, 116.2, 115.6, 115.0, 64.5, 32.5, 30.4−28.9, 26.5, 23.2, 14.2.

Eicosyl Caffeate (13). ^{1}H NMR (acetone-d_6) δ 8.45 (1H, s, OH), 8.21 (1H, s, OH), 7.58 (1H, d, J = 15.9 Hz), 7.20 (1H, d, J = 2.1 Hz), 7.09 (1H, dd, J = 8.1, 2.1 Hz), 6.91 (1H, d, J = 8.1 Hz), 6.32 (1H, d, J = 15.9 Hz), 4.18 (2H, t, J = 6.6 Hz), 1.72 (2H, m), 1.32 (34H, br s), 0.92 (3H, t, J = 6.9 Hz). ^{13}C NMR (acetone-d_6) δ 167.3, 148.5, 146.1, 145.3, 127.5, 122.3, 116.2, 115.6, 115.0, 64.5, 32.5, 30.4−28.9, 26.5, 23.2, 14.2.

Isopropyl Ferulate (16). ^{1}H NMR ($CDCl_3$) δ 7.59 (1H, d, J = 15.9 Hz), 7.06 (1H, dd, J = 8.4, 2.0 Hz), 7.00 (1H, d, J = 2.1 Hz), 6.91 (1H, d, J = 8.4 Hz), 6.27 (1H, d, J = 15.9 Hz), 5.13 (1H, m), 3.91 (3H, s, OMe), 1.30 (6H, d, J = 6.0 Hz). ^{13}C NMR ($CDCl_3$) δ 166.8, 147.8, 146.7, 144.4, 127.0, 123.0, 116.1, 114.6, 109.2, 67.6, 55.9, 21.9.

***n*-Butyl Ferulate (17).** ^{1}H NMR ($CDCl_3$) δ 7.62 (1H, d, J = 15.0 Hz), 7.19 (1H, dd, J = 8.0, 2.0 Hz), 7.05 (1H, d, J = 2.0 Hz), 6.98 (1H, d, J = 8.0 Hz), 6.32 (1H, d, J = 15.0 Hz), 4.22 (2H, t, J = 6.0 Hz), 3.98 (3H, s, OMe), 1.73 (2H, m), 1.45 (2H, m), 1.0 (3H, t, J = 6.0 Hz). ^{13}C NMR ($CDCl_3$) δ 167.4, 147.9, 146.7, 144.6, 127.0, 123.0, 115.6, 114.7, 109.3, 64.3, 55.9, 30.8, 19.2, 13.7.

Isobutyl Ferulate (18). ^{1}H NMR ($CDCl_3$) δ 7.62 (1H, d, J = 15.0 Hz), 7.19 (1H, dd, J = 8.0, 2.0 Hz), 7.05 (1H, d, J = 2.0 Hz), 6.98 (1H, d, J = 8.0 Hz), 6.32 (1H, d, J = 15.0 Hz), 4.01 (2H, d, J = 6.0 Hz), 3.98 (3H, s, OMe), 2.0 (1H, m), 0.97 (6H, d, J = 6.0 Hz). ^{13}C NMR ($CDCl_3$) δ 167.4, 147.9, 146.7, 144.6, 127.0, 123.0, 115.6, 114.7, 109.3, 70.5, 55.9, 27.8, 19.1.

***n*-Octyl Ferulate (21).** ^{1}H NMR ($CDCl_3$) δ 7.62 (1H, d, J = 15.0 Hz), 7.19 (1H, dd, J = 8.0, 2.0 Hz), 7.03 (1H, d, J = 1.8 Hz), 6.91 (1H, d, J = 8.4 Hz), 6.29 (1H, d, J = 15.9 Hz), 4.18 (2H, t, J = 6.6 Hz), 3.92 (3H, s, OMe), 1.67 (2H, m), 1.29 (10H, br s), 0.88 (3H, t, J = 6.6 Hz). ^{13}C NMR ($CDCl_3$) δ 167.4, 147.9, 146.7, 144.6, 127.0, 123.0, 115.6, 114.7, 109.3, 64.6, 55.9, 31.8, 29.2, 29.18, 26.0, 22.6, 14.1.

Isooctyl Ferulate (22). ^{1}H NMR ($CDCl_3$) δ 7.62 (1H, d, J = 15.0 Hz), 7.19 (1H, dd, J = 8.0, 2.0 Hz), 7.05 (1H, d, J = 2.0 Hz), 6.98 (1H, d, J = 8.0 Hz), 6.32 (1H, d, J = 15.0 Hz), 5.01 (1H, m), 3.98 (3H, s, OMe), 1.73 (1H, m), 1.45 (1H, m), 1.28 (8H, br s), 1.24 (3H, d, J = 6.0 Hz), 0.92 (3H, t, J = 6.0 Hz). ^{13}C NMR ($CDCl_3$) δ 167.0, 147.8, 146.7, 144.4, 127.1, 123.0, 116.1, 114.6, 109.2, 71.0, 55.9, 36.0, 31.7, 29.1, 25.4, 22.6, 20.1, 14.1.

Dodecyl Ferulate (23). ^{1}H NMR ($CDCl_3$) δ 7.62 (1H, d, J = 15.9 Hz), 7.07 (1H, dd, J = 8.4, 2.1 Hz), 7.03 (1H, d, J = 2.1 Hz), 6.91 (1H, d, J = 8.1 Hz), 6.29 (1H, d, J = 15.9 Hz), 4.19 (2H, d, J = 6.6 Hz), 3.92 (3H, s, OMe), 1.68 (4H, m), 1.26 (14H, br s), 0.88 (3H, t, J = 6.6 Hz). ^{13}C NMR ($CDCl_3$) δ 167.3, 147.8, 146.7, 144.6, 127.0, 123.0, 115.6, 114.6, 109.2, 64.6, 55.9, 31.8, 29.6, 29.5, 29.3, 29.2, 28.7, 26.0, 22.6, 14.1.

Eicosyl Ferulate (26). ^{1}H NMR ($CDCl_3$) δ 7.59 (1H, d, J = 15.9 Hz), 7.06 (1H, dd, J = 8.4, 2.1 Hz), 7.02 (1H, d, J = 2.0 Hz), 6.96 (1H, d, J = 8.4 Hz), 6.27 (1H, d, J = 15.9 Hz), 4.16 (2H, t, J = 6.6 Hz), 3.90 (3H, s, OMe), 1.67 (2H, m), 1.23 (34H, br s), 0.86 (3H, t, J = 6.0 Hz). ^{13}C NMR ($CDCl_3$) δ 167.4, 147.9, 146.7, 144.6, 127.0, 123.0, 115.6, 114.7, 109.2, 64.6, 55.9, 31.9, 29.7, 29.5, 29.4, 29.3, 28.8, 26.0, 22.7, 14.1.

RESULTS

The major hydroxycinnamic acids present in cereals are ferulic acid, *p*-coumaric acid, and sinapic acid (*1*). The anecdotal reports on the health benefits of these compounds are primarily due to their antioxidant capacity alone. Therefore, we have synthesized a series of alkyl esters of caffeic and ferulic acids to study the impact of alkyl chain lengths of these esters and their tumor cell proliferation, COX enzyme, and lipid peroxidation inhibitory activities. In tumor cell proliferation inhibitory assays, caffeic acid esters were more active than the ferulates. For example, dodecyl (**10**) and hexadecyl (**11**) caffeates inhibited colon cells by 89% and 88%, respectively, at 20 μg/mL (**Figure 2A**). A similar trend was observed for these compounds on all other cell lines tested; dodecyl (**10**) and hexadecyl (**11**) caffeates gave 82% and 75% inhibition on breast tumor cells; 80% and 77% inhibition on lung tumor cells; 79% and 52% on CNS tumor cells; and 67% and 72% inhibition on AGS tumor cells (**Figure 2B**−**E**). The IC_{50} values observed for compounds **10** were 4.35 (breast), 5.37 (lung), 2.46 (colon), 8.09 (CNS), and 7.57 (AGS) μg/mL; and for compound **11**, 9.65 (breast), 8.62 (lung), 5.78 (colon), 12.04 (CNS), and 16.55 (AGS) μg/mL. However, caffeates **3**−**8** showed only 40−50% inhibition on colon cancer cell lines at 20 μg/mL. A similar growth inhibitory effect was also observed for breast and lung cancer cell lines. These compounds, **3**−**8**, were also less effective on gastric and CNS tumor cell lines as indicated by <30% growth inhibition.

Among ferulate esters, octyl (**21**) and dodecyl (**23**) esters of ferulic acids were the most active and inhibited the growth of breast tumor cells by 78% and 86%; of lung tumor cells by 27% and 77%; of colon tumor cells by 80% and 93%; of CNS tumor cells by 48% and 79%; and of AGS tumor cells by 81% and 87%, respectively (**Figure 2A**−**E**). The IC_{50} values observed for compound **21** were 17.05 (breast), 11.0 (lung), 4.29 (colon), 14.63 (CNS), and 18.67 (AGS) μg/mL. Similarly, dodecyl ferulate (**23**) exhibited IC_{50} values of 7.45, 7.76, 3.5, 12.21, and 13.46 μg/mL on breast, lung, colon, CNS, and AGS

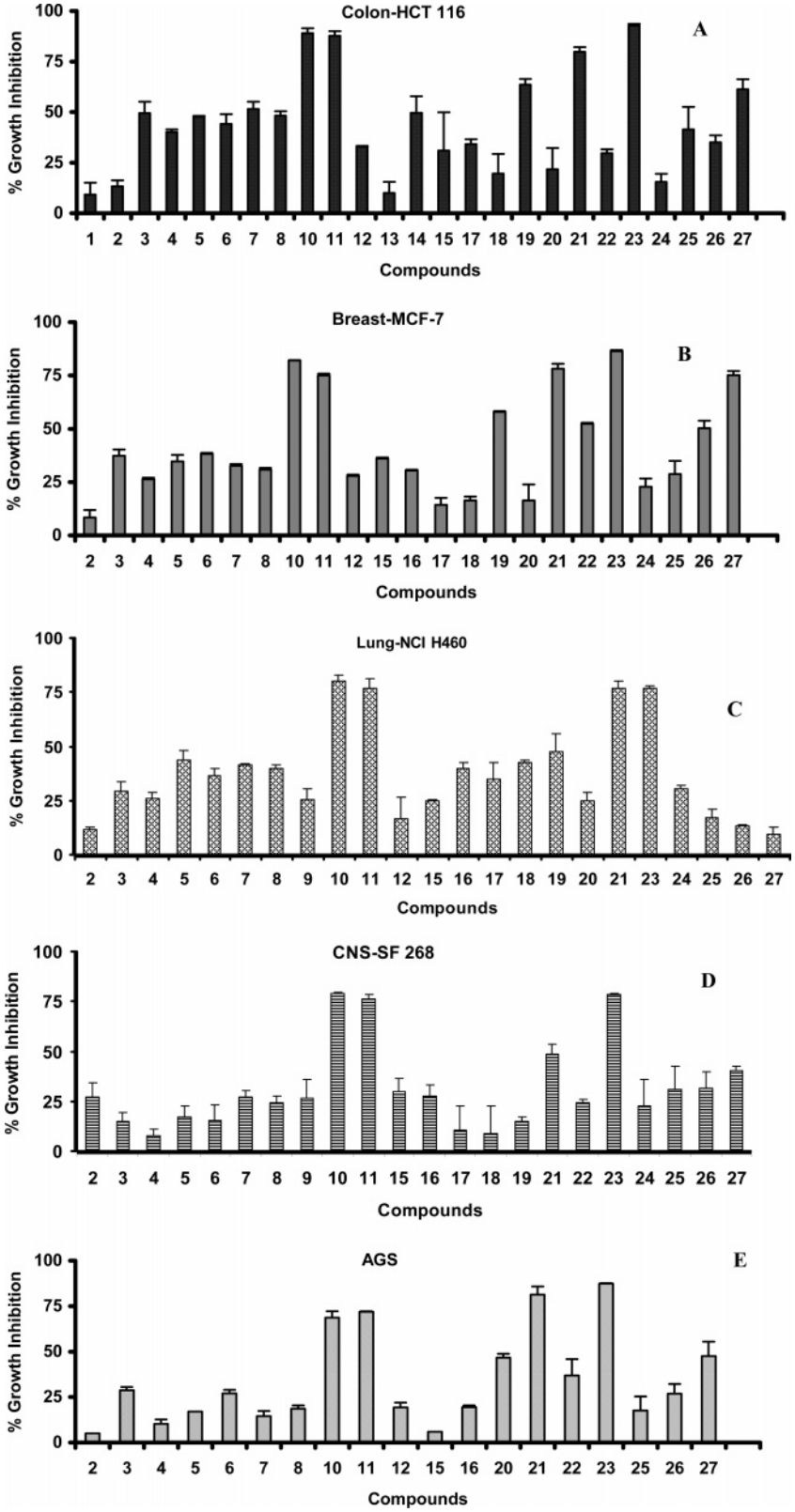

Figure 2. Effect of alkyl caffeates and ferulates on growth of (**A**) colon (HCT-116), (**B**) breast (MCF-7), (**C**) lung (NCI H-460), (**D**) CNS (SF-268), and (**E**) gastric (AGS) human tumor cells as determined by MTT assay. Compounds were incubated for 48 h at 20 μg/mL. Values represents mean of two independent experiments assayed in triplicate ± SD.

tumor cells, respectively. Compounds **19** and **27** were also moderately active against breast and colon tumor cells as shown by 55−80% growth inhibition. However, these compounds were not significantly active on other tumor cell lines tested. The percent growth inhibition for other ferulates tested in this assay varied from 10% to 40%.

In the COX enzyme inhibitory assay, ferulic and caffeic acid alkyl esters inhibited both COX-1 and COX-2 enzymes at 25 μg/mL. The caffeates **5**, **7** and **9** were the most active and inhibited COX-1 enzyme by 79%, 92%, and 83% and COX-2 enzyme by 59%, 64%, and 61%, respectively (**Figure 3A**). Both isopropyl and *n*-propyl esters of caffeic acid did not inhibit

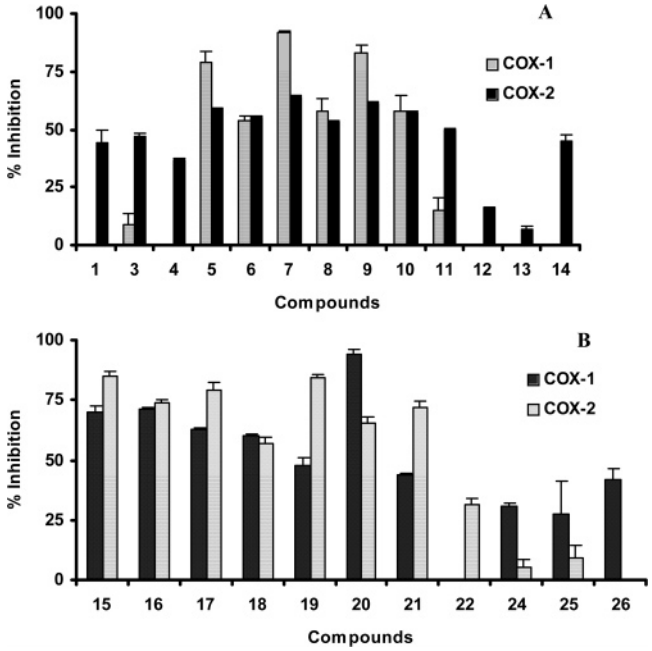

Figure 3. Inhibition of COX-1 and -2 enzymes by alkyl esters of (**A**) caffeic acid and (**B**) ferulic acid at 25 μg/mL. DMSO was used as solvent control. Percent inhibition was calculated with respect to DMSO control. Vertical bars represent average of two experiments ± SD.

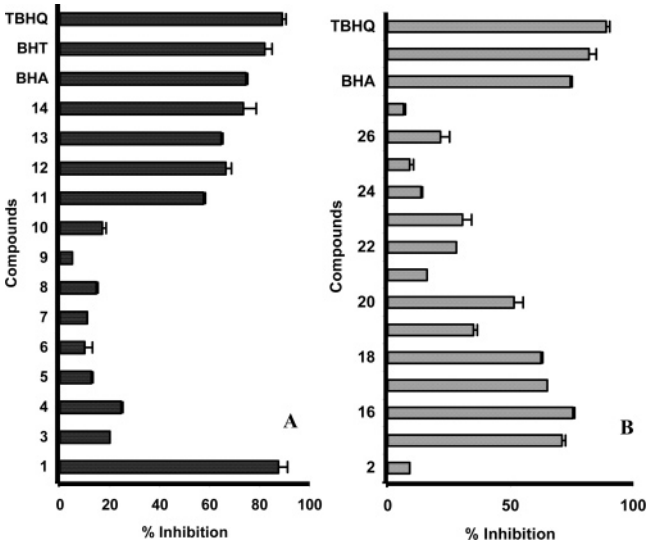

Figure 4. Inhibition of Lipid peroxidation by alkyl esters of (**A**) caffeic acid and (**B**) ferulic acid at 5 μg/mL. Commercial antioxidants BHA, BHT and TBHQ were used as positive controls and tested at 1 μM concentration. Lipid peroxidation was initiated by Fe^{2+} and the rate of decrease in fluorescence intensity was monitored over 21 min. The percent inhibition was calculated with respect to solvent control (DMSO) and values represent mean ± SD ($n = 2$).

COX-1 enzyme but inhibited COX-2 by 47% and 37%, respectively (**Figure 3A**). The isoalkyl esters of caffeic acid, **6** and **8**, were less active than their corresponding *n*-alkyl esters and showed 54% and 58% inhibition of COX-1 and 55% and 53% of COX-2 enzyme, respectively. The parent compound caffeic acid (**1**) and its propyl esters, **3** and **4**, showed selective COX-2 enzyme inhibition by about 35−45%. Similarly, compounds **11** and **14** also inhibited COX-2 enzyme selectively by 50% and 45%, respectively (**Figure 3A**). That is, the ferulates up to chain length C_8 showed potent COX-2 enzyme inhibition. Although nonselective, the *n*-propyl (**15**), *n*-butyl (**17**), *n*-amyl (**19**), and *n*-octyl (**21**) esters of ferulic acids inhibited COX-2 enzyme by 85%, 79%, 84%, and 71%, respectively. The isopropyl (**16**), isobutyl (**18**), isoamyl (**20**), and isooctyl (**22**) esters inhibited COX-2 enzyme by 74, 56, 65 and 31%, respectively (**Figure 3B**). The octadecyl ester of ferulic acid (**23**) did not inhibit the activity of COX-1 and -2 enzymes. However, compounds **24**−**26** showed activity more selective toward COX-1 enzyme inhibition by 35−45% (**Figure 3B**). At 50 μg/mL concentration, compounds **15**−**21** attained 100% COX enzyme inhibitory activity (data not shown). The nonsteroidal antiinflammatory drugs (NSAIDs) aspirin (180 μg/mL), ibuprofen (2.52 μg/mL), and naproxen (2.06 μg/ mL) inhibited COX-1 enzyme by 61%, 53%, and 79% and COX-2 enzyme by 24%, 5%,9 and 96%, respectively. Vioxx, a selective COX-2 inhibitor, was tested and showed 76% inhibition at 1.67 μg/mL concentration. The varying concentrations of positive controls used were necessary to keep the COX enzyme inhibition between 50% and 100%.

Compounds **1**−**28** were also evaluated for the inhibition of Fe^{2+}-induced lipid peroxidation by using large unilamellar vesicles (LUVs). At 5 μg/mL concentration, short-chain ferulates and long-chain caffeates showed strong lipid peroxidation inhibitory activity. Interestingly, caffeic acid inhibited lipid peroxidation by 85%, whereas ferulic acid showed little or no activity. The positive controls butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tetrabutylhydroxyquinone (TBHQ) were also tested in this assay at 1 μg/mL and showed 74%, 82%, and 89% lipid peroxidation inhibition, respectively. The inhibition observed for compounds **3**−**10** was below 25%. However, compounds **11**−**14** with chain lengths of C_{16}, C_{18}, C_{20}, and C_{22} inhibited lipid peroxidation by 58%, 67%, 65%, and 74%, respectively (**Figure 4A**). In contrast, short-chain ferulates **15**−**18** were more active and showed 71%, 76%, 65%, and 63% inhibition, respectively. The percent inhibition for compounds **21**−**27** was in the range of 10−30% (**Figure 4B**).

DISCUSSION

The present study was undertaken to compare the tumor cell proliferation, cyclooxygenase, and lipid peroxidation inhibitory activities of feruloyl and caffeoyl esters. The results indicated that caffeic acid esters are more active than the ferulic acid esters in inhibiting the cell proliferation of colon and breast cancer cell lines. Both caffeic and ferulic acids, the phenolic acids, were unable to inhibit the growth of all tumor cell lines tested at 20 μg/mL. The esterification of these phenolic acids with alkanols enhanced the cell proliferation inhibitory activity. The isoalkyl esters **4**, **6**, and **8** were slightly less active than the corresponding *n*-alkyl esters of caffeic acid on colon cancer cells. Similar effect was observed with ferulic acid esters on this cell line. The gastric (AGS) tumor cells were least affected by ferulates and caffeates. Among the caffeates, dodecyl (**10**) and hexadecyl (**11**) esters were more active and inhibited the proliferation of all tumor cell lines tested to the highest level (**Figure 2**). Similarly, octyl (**21**) and dodecyl (**23**) esters of ferulic acid were more active among the ferulates tested in the cell proliferation assay (**Figure 2**).

The conformation, stability, and hydrophilicity changes of these phenolic esters have been reported as a function of chain length (*22*, *23*). These factors could also play a significant role in the tumor cell proliferation inhibitory activity demonstrated by these esters. Our results suggest that the most active caffeates were C_{12} and C_{16} and the most active ferulates were C_8 and C_{12} on tested tumor cell lines. Recent study on some of the

caffeates and gallates showed the number of hydroxyl groups in the phenol moiety was also important in defining the antiproliferative activity of these compounds (*24*). However, we did not observe much difference in the activity profile for ferulic and caffeic acid esters in our study. Both ferulic and caffeic acid esters inhibited COX-1 and -2 enzymes (**Figure 3**). Although ferulic acid did not inhibit COX enzymes, caffeic acid showed specific COX-2 inhibition (**Figure 3A**). The caffeates with chain lengths between C_4 and C_{12} showed both COX-1 and -2 enzyme inhibition, and specificity toward COX-2 increased at chain lengths > C_{12}. Among the ferulates, the COX inhibitory activity decreased considerably for esters with chain length > C_8. This may be due to the conformation of these compounds, which impacts their interaction with the enzymes. The lipid peroxidation assay revealed that the caffeic acid esters with chain lengths up to C_{12} were less inhibitory as compared to the long-chain esters (C_{16}−C_{22}) (**Figure 4A**). This indicated that hydrophilicity of these compounds may play a significant role in the antioxidant activity of these compounds. In contrast, the ferulic acid derivatives **15**−**23** (C_3−C_{12}) were more active than their long-chain counterparts (**22**−**27**) (**Figure 4B**). The conformational rigidity of the ferulates was one of the factors reported for their antioxidant activity (*23*). Although it is difficult to explain this trend in activity by examining the chemical structures, the trend in activity may be explained on the basis of conformational changes and interaction with the enzyme under assay conditions.

Ferulic and caffeic acids are ubiquitous in nature, and the alkyl esters of ferulic acid are components in a number of dietary products (*25*, *26*). Several caffeic acid alkyl esters have been reported to possess a wide array of biological activities (*24*, *27*). As reported earlier, reactive oxygen species play a significant role in the regulation of tumor cell growth and expression of COX-2 enzyme (*28*, *29*). Also, the overexpression of COX-2 enzyme and increased production of prostaglandins in several types of cancer, including colon cancer, has been reported (*30*). Epidemiological and experimental studies have shown that the inhibition of COX enzymes could reduce the risk of colon carcinogenesis (*29*). Our study revealed that both caffeic and ferulic acid esters inhibited tumor cell proliferation, COX enzyme, and lipid peroxidation in vitro. Hence, the presence of these lipid peroxidation inhibitory phenolic esters in food products may offer nonnutritional health benefits. Further research is warranted to determine the clinical efficacy and dosage of these dietary phenolics.

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