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Isolation and Identification of Phase II Enzyme-Inducing Agents from Nonpolar Extracts of Green Onion (*Allium* spp.)

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The objective of the study was to isolate and identify potential cancer preventive constituents from green onion based on the ability to induce quinone reductase (QR, a representative phase II enzyme) in murine hepatoma cells (Hepa 1c1c7). Crude nonpolar solvent extracts were prepared from freeze-dried green onion by sequential refluxing with hexane and then ethyl acetate, followed by liquid−liquid extraction. Active fractions were subjected to the Hepa 1c1c7 bioassay-guided steps of flash chromatography, thin layer chromatography (TLC), and high-pressure preparative liquid chromatography (HPLC) to afford pure isolates capable of inducing QR. Multiple fractions were active in inducing QR. Five pure compounds were isolated from active fractions and identified using spectroscopic methods; these were *p*-hydroxyphenethyl *trans*-ferulate (**1**), 5,6-dimethyl-2-pyridinecarboxylic acid (**2**), ferulic acid (**3**), 1-(6-hydroxy-[3]pyridyl)-propan-1-one (**4**), and *N-trans*-feruloyl 3-*O*-methyldopamine (**5**). *p*-Hydroxyphenethyl *trans*-ferulate (**1**) doubled QR specific activity in Hepa 1c1c7 cells at a level of 2.1 μg/mL (6.6 μM).

KEYWORDS: Green onion; cancer prevention; phase II enzyme; quinone reductase; Hepa 1c1c7 cells

INTRODUCTION

The complement of phase I and II drug-metabolizing enzymes is an important system that the human body has developed to protect itself from environmental toxins. Xenobiotic metabolism by phase I enzymes (e.g., cytochrome P450s) may yield either inactive detoxification products or highly reactive, electrophilic carcinogens that may react with nucleophilic centers such as DNA to initiate carcinogenesis (*1*). Products of phase I metabolism can be substrates for phase II enzymes (e.g., NAD(P)H:quinone reductase, glutathione-*S*-transferase, and UDP−glucuronosyltransferase) and transformed to easily excretable inert products (*2*). An individual's risk of chemical carcinogenesis is largely controlled by the balance between phase I carcinogen-activating enzymes and phase II detoxifying enzymes (*3*). Many lines of evidence have supported the concept that induction of xenobiotic metabolizing enzymes, particularly phase II enzymes, affords protection against neoplastic effects of chemical carcinogens (*4*), and phase II enzyme induction has emerged as an important strategy for chemoprevention (*5*).

NAD(P)H:(quinone acceptor) oxidoreductase 1 (EC 1.6.99.2) (QR), a phase II enzyme, is a flavoprotein that catalyzes the obligatory two-electron reduction of a wide range of substrates including quinones, quinone-imines, glutathionyl-substitued naphthoquinones, dichlorophenolindolphenol, methylene blue, and azo and nitro compounds (*6*, *7*). QR directly detoxifies harmful quinones (e.g., menadione) to yield stable, inactive hydroquinones (*8*) and also acts as an antioxidant enzyme to maintain endogenous antioxidants coenzyme Q (CoQ) and vitamin E in their reduced and active forms (*9*, *10*). QR deficiency in humans is associated with increased risk of leukemia, specifically acute myelogenous leukemia (*11*−*13*). Studies using QR-null mice deficient in QR RNA and protein (*14*) demonstrated protective effects of QR against benzo(*a*)-pyrene- (*15*) and DMBA-mediated carcinogenesis (*16*), as well as benzene-induced toxicity in humans and mice (*17*).

Onions have been used as an important flavoring vegetable worldwide for many centuries and rank second in production to tomatoes among horticultural crops (*18*). Besides being useful in traditional medicine, onions have been shown to possess anticarcinogenic (*19*−*21*), cardiovascular protective (*22*, *23*), antiasthmatic (*24*), and antibiotic (*25*) activities. Most commercial green onions in North America and Europe are young and immature plants of cultivars of the common onion (*Allium cepa*), harvested while the tops are still green and before a large bulb has formed (*26*).

Compared with mature bulb onions and garlic, green onions have been much less studied in terms of their bioactivities. However, crude acetonitrile extracts of green onions were found to be the most potent *in vitro* inducer of QR among crude extracts of 24 vegetables, including broccoli, a rich source of a potent inducer, sulforaphane, capable of doubling QR *in vitro* at ∼0.2 μM (*27*, *28*). Also, a green onion-based "kimchi" was

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a more effective inducer of glutathione S-transferase in rodents than other vegetable-based kimchis studied (*29*).

The compounds responsible for the *in vitro* and *in vivo* effects of onion preparations described are not known. Thus, the objective of the present study was to use a cellular bioassay to guide the isolation and identification of phase II enzyme-inducing agents from organic extracts of green onion.

MATERIALS AND METHODS

Materials and General Experimental Procedures. *trans*-4-Hydroxy-3-methyl-cinnamic acid was purchased from Aldrich Chemical Co. (Milwaukee, WI). α-Minimum essential medium (with L-glutamine, without ribonucleosides and deoxyribonucleoside; MEM), trypsin-EDTA (0.25% trypsin with EDTA-4Na), fetal bovine serum (certified), and penicillin−streptomycin were from Gibco (Grand Island, NY). All other chemicals were from Sigma Chemical Co. (St. Louis, MO). Freeze-dried green onion was purchased as a single lot from Van Drunen Farm (Momence, IL) and stated by the supplier to be comprised of *Allium cepa* (no certification was provided).

TLC was performed on Whatman silica gel (60 Å) UV_{254} plates (layer thickness, 250 μm for analytical and 1000 μm for preparative) with compounds visualized under UV_{254nm} or by iodine staining (Aldrich, Milwaukee, WI) or both. Silica gel (60 Å, 230−400 mesh ASTM; Fisher Scientific, Fair Lawn, NJ) and C18 reverse phase silica gel (60 Å, 230−400 mesh ASTM; EMD Chemical Inc., Gibbstown, NJ) were used for flash column chromatography conducted with Chromaflex columns (Kontes, Vineland, NJ) equipped with a pump (Cole Palmer, Vernon Hills, IL), a model 160 gradient former (ISCO, Lincoln, NE), and a fraction collector. All solvents used for chromatographic separations were purchased from Fisher Scientific (Fair Lawn, NJ). HPLC analysis was conducted using a 250 mm × 4.6 mm i.d., 5 μm, Discovery C18 column (Supelco, Bellefonte, PA) on an Agilent (Foster City, CA) series 1100 system equipped with a diode array detector. Preparative HPLC was carried out on an ISCO (Lincoln, NE) system (model 2300 pumps, V^4 detector) using a 250 mm × 21.2 mm i.d., 5 μm, Discovery C18 column (Supelco, Bellefonte, PA) with peak analysis by an integrator.

Induction of Quinone Reductase (QR). A bioassay based on cultured murine hepatoma cells (Hepa 1c1c7; ATCC, Rockville, MD) was used to assess QR induction essentially as described earlier (*27*). Hepa 1c1c7 cells were grown for 24 h in 96 well plates (5000 cells per well) in 150 μL of MEM medium supplemented with 10% fetal bovine serum (treated with activated charcoal to remove any traces of endogenous QR inducers) and antibiotics at 37 °C in 5% CO_2 in air. Test isolates were added (in 150 μL of complete medium), and cells were induced for an additional 48 h. Isolates with limited aqueous solubility were dispersed in MEM containing 0.1% of hydroxylpropyl β-cyclodextrin or DMSO prior to adding to the cells (final concentration of DMSO was ≤0.5%). No effects on QR induction were observed in control samples incubated with hydroxylpropyl β-cyclodextrin or DMSO alone or in combination.

After culture medium was decanted, cells were lysed by adding 50 μL of saturated digitonin aqueous solution in 2 mM EDTA, pH 7.8, and then incubating at 37 °C for 20 min with gentle shaking. For the QR assay, a standard assay cocktail (containing the essential components of FAD, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP, 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide, and menadione) was prepared and added (*27*) in a final volume of 200 μL to each well. QR activity was determined by measuring absorbance of the reduced tetrazolium dye over a 10-min period using a SPECTRA MAX plus optical microtiter plate scanner (Molecular Devices, Sunnyvale, CA) set at 490 nm. A duplicate plate was prepared for cell protein (viability) assessment and to facilitate enzyme specific activity calculation. The concentration required for doubling the specific activity of QR (CD value) relative to nontreated control cells was used as an indicator of inducer potency.

Glutathione-*S*-Transferase (GST) Activity Assay. Hepa 1c1c7 cells were cultured, treated with test samples, and then lysed in the same way as described in the previous section. GST activity was measured by an established method adapted to a 96-well plate (*30*). A stock solution (150 μL) containing 1.33 mM of 1-chloro-2,4-dinitrobenzene, 1.33 mM of glutathione (reduced), and 100 mM PBS (pH 6.4) was added to each cell lysate in the microtiter plate. The absorbance of the conjugate formed was recorded over a 10-min period using an optical microtiter plate scanner set at 340 nm. A duplicate plate was prepared for cell protein (viability) assessment and to facilitate enzyme specific activity calculation.

Cell Protein (Viability) Assessment. Culture medium was decanted from a replicate 96 well plate, and induced cells were assayed for protein by incubation with 100 μL of crystal violet (0.2% in 2% ethanol) for 10 min (*27*). After free dye was washed off with lukewarm tap water, bound dye was solubilized by adding 150 μL of 0.5% (w/v) SDS solution in ethanol/water (1:1). After incubation for 1 h in a shaker oven at 37 °C, absorbance was measured at 610 nm. An IC_{50} was calculated as the concentration of isolate required to decrease cell protein (cell viability) by 50% relative to untreated control cells.

Statistical Analysis. Data are reported as means ± standard deviations of at least three experiments. The differences among mean values were evaluated by a two-tailed Student's *t*-test.

Extraction and Isolation. *Crude Extracts.* Freeze-dried green onion (1500 g) was sequentially extracted by refluxing with hexane and then ethyl acetate using an oversize Soxhlet apparatus (extraction chamber 10 cm × 40 cm), where each extraction lasted for 8 h. After drying *in vacuo* at 50 °C, the hexane extract (HX, 31.4 g) was suspended in 500 mL of 80% methanol (20% aqueous) and then partitioned with an equal amount of hexane 5 times to afford a methanol-partitioned fraction (HX-ME, 4.95 g) and a hexane-partitioned fraction (HX-HX, 26.0 g). In a similar fashion, the ethyl acetate extract (EA, 52.9 g) was partitioned between hexane and 80% methanol to yield a hexane-partitioned fraction (EA-HX, 17.1 g) and a methanol-partitioned fraction (EA-ME, 35.6 g). Subsequent QR bioassay in the Hepa 1c1c7 cell line revealed that only the HX-ME and EA-ME fractions had low enough CD values (≤20 μg/mL) to warrant further fractionation.

Fractionation of Partitioned Hexane Crude Extract (HX-ME). The HX-ME isolate was subjected to flash chromatography using silica gel and a five-step gradient of 2.5%, 5%, 10%, 30%, and 100% methanol in methylene chloride at flow rate of 15 mL/min. Each step gradient was run for 54 min. Fractions were collected in 45 mL aliquots and then pooled based on what was judged to be resolved groups of eluting material with the assistance of spectrscopic data collected between 210 to 340 nm (data not shown). Eight pooled fractions were obtained, and six of them had potent QR-inducing power (CD < 10 μg/mL). Chromatographic profiling to ascertain relative complexity of these eight fractions was achieved by reverse phase HPLC using gradients of acetonitrile in 1% acetic acid (data not shown).

Fraction 3 (395 mg) of the HX-ME isolate was further resolved by reverse phase flash chromatography on C18 silica gel with a linear gradient from 20% to 70% acetonitrile in 1% acetic acid, a flow rate 16 mL/min, and run time of 500 min to give two major subfractions, 3A and 3B. Subfraction 3A was further purified by silica gel preparative TLC with 50% acetone in ethyl acetate as developing solvent, giving a major band at R_f 0.22, and the resulting isolate afforded compound **1** (5 mg) after a final purification step using reverse phase preparative HPLC with isocratic 35% acetonitrile in 1% acetic acid and flow rate of 7 mL/min.

Fraction 8 recovered from the crude HX-ME isolate by elution with 100% methanol (828 mg) was fractionated by flash chromatography on C18 silica gel with a linear gradient from 2% to 30% acetonitrile in 1% acetic acid, flow rate of 16 mL/min, and run time of 300 min to produce one major subfraction. Further purification by reverse phase preparative HPLC with isocratic 2% acetonitrile in 1% acetic acid and flow rate of 7 mL/min afforded compound **2** (3 mg).

Fractionation of Partitioned Ethyl Acetate Crude Extract (EA-ME). Normal phase silica gel flash chromatography was used to resolve the EA-ME isolate using the same conditions described for the HX-ME isolate. Six pooled fractions were obtained, and fraction 3 eluted by 5% MeOH was subjected to further separation based on its relatively high QR-inducing potency, sufficient abundance (1.9 g), and relative lack of complexity in the HPLC profile. Reverse phase flash chromatography on C18 silica gel with a linear gradient from 2% to 30% of

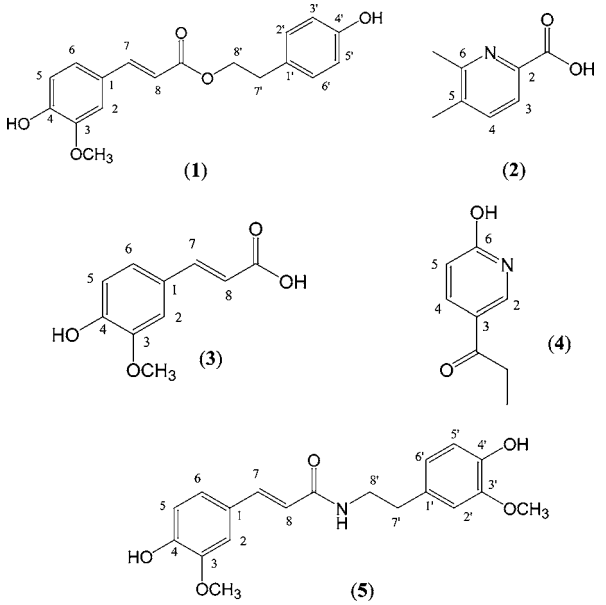

Figure 1. Chemical structures of compounds **1**–**5**.

acetonitrile in 1% acetic acid within 288 min at a flow rate of 8 mL/min of fraction 3 afforded three subfractions, 3A, 3B, and 3C. Fractions 3B and 3C were further resolved by reverse phase preparative HPLC with isocratic 15% acetonitrile in 1% acetic acid for 3B, and isocratic 25% acetonitrile in 1% acetic acid for 3C at a flow rate 7 mL/min, to obtain compound **3** (10 mg) and compounds **4** (2.5 mg) and **5** (17 mg) from these respective subfractions.

Structure Identification. Proton NMR spectra were obtained in DMSO-d_6 (unless otherwise noted) on Varian ui400 (400 MHz) and ui500 (500 MHz) spectrometers at 25 °C. ^{13}C-Spectra were obtained in DMSO-d_6 (unless otherwise noted) on a Varian ui500 spectrometer at 25 °C. Two-dimensional NMR experiments (COSY, HMQC, HSQC, and HMBC) were performed in the same solvent as were ^{13}C experiments on the Varian ui500 spectrometer. All NMR experiments were performed using standard pulse sequences.

The EI-MS experiments were carried out on a Micromass (Waters, Milford, MA) 70-250-S magnetic sector mass spectrometer equipped with an Opus data system. Nominal mass spectra were obtained using a direct sample probe, and the magnet was scanned from *m*/*z* 1000 to *m*/*z* 35 at 70 eV, source temperature 230 °C, external calibration perfluorokerosene-H [PFK]. The HREI exact mass measurements were conducted on the same instrument (6000 resolution, linear voltage) using PFK peaks as known masses. The ESI-MS were performed on either a Waters LCT electrospray-TOF instrument or a Waters Autospec Ultima magnetic sector instrument equipped with an electrospray interface. HRESI exact mass measurements were obtained on the Waters Autospec Ultima (linear voltage scanning) using sodiated poly(ethylene glycol) peaks as known masses. All MS experiments were carried out at the mass spectrometry facility of the Department of Chemistry at the University of Michigan (Ann Arbor, MI). The structures of the compounds isolated by these protocols are shown in **Figure 1**.

Spectroscopic and Spectrometric Data of Isolated Compounds. *p-Hydroxyphenethyl trans-Ferulate (**1**).* HREI *m*/*z*: 314.1154 (calcd for $C_{18}H_{18}O_5$, 314.1154). EI (70 eV) *m*/*z* (rel. int.): 314 M^+ (3), 194 [M − (*p*-hydroxyphenethyl moiety) + H]$^+$ (100), 177 [feruloyl moiety − O]$^+$ (26), 145 (21), 121 [*p*-hydroxyphenethyl moiety]$^+$ (10), 120 [*p*-hydroxyphenethyl moiety − H]$^+$ (61). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.52 (1H, *d*, *J* = 16 Hz, H-7), 7.31 (1H, *d*, *J* = 2.0 Hz, H-2), 7.10 (1H, *dd*, *J* = 8.4, 2.0 Hz, H-6), 7.07 (2H, *d*, *J* = 8.4 Hz, H-2′ and H-6′), 6.78 (1H, *d*, *J* = 8.0 Hz, H-5), 6.69 (2H, *d*, *J* = 8.4 Hz, H-3′ and H-5′), 6.44 (1H, *d*, *J* = 16 Hz, H-8), 4.25 (2H, *t*, *J* = 7.0 Hz, H-8′), 3.81 (3H, *s*, Me), 2.83 (2H, *t*, *J* = 7.0 Hz, H-7′). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 166.5 (COO), 155.8 (C-4′), 149.4 (C-4), 147.9 (C-3), 145.0 (C-7), 129.6 (2C, C-2′ and C-6′), 127.9 (C-1′, 125.4 (C-1), 123.2 (C-6), 115.4 (C-5), 115.1 (2C, C-3′ and C-5′), 114.2 (C-8), 111.1 (C-2), 64.5 (C-8′), 55.6 (Me), 33.6 (C-7′).

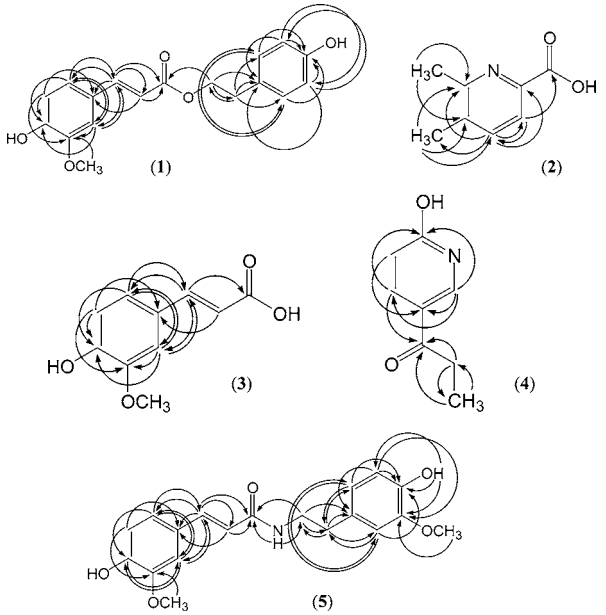

Figure 2. Significant proton–carbon long-range correlations observed from HMBC spectra.

*5,6-Dimethyl-2-pyridinecarboxylic Acid (**2**).* HREI *m*/*z*: 151.0623 (calcd for $C_8H_9NO_2$, 151.0633). EI (70 eV) *m*/*z* (rel. int.): 151 M^+ (17), 134 [M − OH]$^+$ (3), 107 [M − COO]$^+$ (100), 106 [M − COOH]$^+$ (58), 79 (23). ESI *m*/*z*: 152 [M + H]$^+$. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.96 (1H, *s*, *J* = 7.5 Hz, H-3), 7.64 (1H, *d*, *J* = 7.5 Hz, H-4), 2.55 (3H, *s*, C6-C*H*$_3$), 2.38 (3H, *s*, C5-C*H*$_3$). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 164.5 (COOH), 156.3 (C-6), 143.0 (C-2), 139.1 (C-4), 137.2 (C-5), 121.3 (C-3), 22.2 (C6-*C*H$_3$), 19.3 (C5-*C*H$_3$).

*Ferulic Acid (**3**).* HREI *m*/*z*: 194.0595 (calcd for $C_{10}H_{10}O_4$, 194.0579). EI (70 eV) *m*/*z* (rel. int.): 194 M^+ (35), 150 (100), 135 (73), 107 (28), 77 (27), 44 (30). ^{1}H NMR (DMSO-d_6, 500 MHz): δ 7.44 (1H, *d*, *J* = 15.5 Hz, H-7), 7.25 (1H, *s*, H-2), 7.05 (1H, *d*, *J* = 8.0 Hz, H-6), 6.78 (1H, *d*, *J* = 8.0 Hz, H-5), 6.36 (1H, *d*, *J* = 15.5 Hz, H-8), 3.81 (3H, *s*, OMe). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 168.2 (COOH), 148.9 (C-4), 147.8 (C-3), 144.0 (C-7), 125.8 (C-5), 122.6 (C-6), 116.0 (C-5), 115.4 (C-8), 111.0 (C-2), 55.6 (OMe) (matched the NMR and MS spectra of a sample of standard ferulic acid).

*1-(6-Hydroxy-[3]pyridyl)-propan-1-one (**4**).* HREI *m*/*z*: 151.0649 (calcd for $C_8H_9NO_2$, 151.0633). EI (70 eV) *m*/*z* (rel. int.): 151 M^+ (39), 123 (50), 122 (56), 104 (22), 95 (65), 94 (100), 76 (22), 57 (16), 44 (25), 39 (25). ^{1}H NMR (DMSO-d_6, 500 MHz): δ 8.16 (1H, *s*, H-2), 7.85 (1H, *d*, *J* = 8.5 Hz, H-4), 7.22 (1H, *d*, *J* = 8.5 Hz, H-5), 3.07 (2H, *q*, *J* = 7.5 Hz, CH_2), 1.06 (3H, *t*, *J* = 7.0 Hz, CH_3). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 200.2 (C═O), 157.8 (C-6), 145.0 (C-3), 137.3 (C-2), 123.2 (C-4), 122.2 (C-5), 29.9 (CH_2), 8.1 (CH_3).

*N-trans-Feruloyl 3-O-Methyldopamine (**5**).* HRESI *m*/*z*: 344.1496 (calcd for $C_{19}H_{22}NO_5$ [M + H]$^+$, 344.1498). EI (70 eV) *m*/*z* (rel. int.): 343 [M + H]$^+$ (7), 194 (17), 193 (17), 177 (44), 150 (100), 145 (25), 117 (11). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.70 (1H, *s*, C4′-OH), 7.97 (1H, *t*, *J* = 5.6 Hz, NH), 7.11 (1H, *d*, *J* = 16 Hz, H-7), 6.97 (1H, *dd*, *J* = 8.4, 1.6 Hz, H-6), 6.78 (1H, *d*, *J* = 8.4 Hz, H-5), 6.77 (1H, *d*, *J* = 1.6 Hz, H-2′), 6.68 (1H, *d*, *J* = 7.6 Hz, H-5′), 6.60 (1H, *dd*, *J* = 8.0, 1.6 Hz, C-6′), 6.43 (1H, *d*, *J* = 16 Hz, H-8), 3,80 (3H, *s*, C3-OMe), 3.74 (3H, *s*, C3′-OMe), 3.36 (2H, *t*, *J* = 7.2 Hz, H-8′), 2.65 (2H, *t*, *J* = 7.4 Hz, H-7′). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 165.2 (C═O), 148.1 (C-4), 147.7 (C-3), 147.3 (C-3′), 144.7 (C-4′), 138.7 (C-7), 130.1 (C-1′), 126.3 (C-1), 121.4 (C-6), 120.6 (C-6′), 119.0 (C-8), 115.5 (C-5), 115.2 (C-5′), 112.7 (C-2), 110.7 (C-2), 55.4 (2C, C3-O*Me* and C3′-O*Me*), 40.4 (C-8′), 34.7 (C-7′).

Proton−carbon long-range correlations obtained by HMBC spectra, important to facilitating identification of the isolated compounds, are shown in **Figure 2**.

Table 1. QR-Inducing Activities of Crude Solvent Extracts and Fractions Therefrom[a]

test sample	CD ± SD (μg/mL)	IC_{50} ± SD (μg/mL)	IC_{50}/CD
	Crude Isolates		
HX-ME	7.3 ± 0.67	15.6 ± 0.8	2.14
HX-HX	852 ± 91.8	>1000	>1.17
EA-ME	20.7 ± 0.9	62.5 ± 5.9	3.02
EA-HX	179 ± 1.5	576 ± 71.2	3.22
	Fractions of HX-ME		
1	2.8 ± 0.8	25.5 ± 0.2	9.11
2	3.1 ± 0.4	35.9 ± 1.6	11.6
3	6.2 ± 0.1	49.8 ± 1.6	8.03
4	27.3 ± 3.8	321 ± 1.2	11.8
5	15.7 ± 1.0	54.6 ± 9.5	3.48
6	5.6 ± 0.3	36.9 ± 3.1	6.59
7	3.8 ± 0.4	3.0 ± 0.3	0.79
8	4.3 ± 1.0	5.9 ± 0.6	1.37
	Fractions of EA-ME		
1	11.1 ± 2.2	31.7 ± 6.5	2.86
2	10.9 ± 2.0	34.3 ± 4.4	3.15
3	16.5 ± 1.0	58.7 ± 0.9	3.56
4	31.5 ± 6.10	79.4 ± 16.7	2.52
5	99.5 ± 9.0	254 ± 28.0	2.56
6	98.4 ± 6.6	119.7 ± 5.2	1.22

[a] CD and IC_{50} were mean ± standard deviation of at least three replicates. All values were significantly different ($p < 0.01$) from the controls by a two-tailed Student's *t*-test. Boldface isolates were subject to further purification efforts.

RESULTS AND DISCUSSION

QR Inducing Activity of Crude Solvent Extracts. Sequential solvent extraction was used to fractionate green onion solids into nonpolar and moderately nonpolar crude extracts (hexane- and ethyl acetate-soluble, respectively). Subsequent solvent partitioning yielded four crude isolates. Greatest enrichment of QR-inducing activity from crude extracts was obtained by partitioning into 80% methanol (**Table 1**). The QR-inducing potency of the HX-ME isolate was greater than that of EA-ME isolate ($p < 0.05$). The HX-ME isolate doubled QR activity at 7.3 μg/mL at a level one-half of the IC_{50} (15.6 μg/mL). The EA-ME isolate was obtained in >7-fold greater abundance than the HX-ME isolate and was also judged suitable for further fractionation and isolation, although its CD value for QR induction was about 3-fold greater (less potent) than that of the HX-ME isolate. The total weight of these two active crude isolates (40.6 g) was about 48% of the total weight of the initial crude extracts, which suggested that sequential solvent extraction and partitioning were effective as initial steps for recovering and concentrating QR-inducing activities. The HX-ME and EA-ME isolates were subjected to further fractionation.

QR-Inducing Activity of Fractions of Crude Solvent Isolates. *Fractionation of the HX-ME Isolate.* Silica flash chromatography with stepwise gradient elution yielded eight fractions from the HX-ME isolate. The cellular QR-inducing bioassay revealed all eight fractions to be QR inducers, many with CD values lower than 10 μg/mL (**Table 1**) with an 86% recovery of loaded material. Six of these active fractions had CD values of a similar magnitude, ranging from 2.8 to 6.2 μg/mL. Combined with observations of cell growth inhibition, fractions 1−3 and 6 doubled QR activity at concentrations where no significant loss of viability was observed (IC_{50}/CD ranged from 6.6 to 11.8 units), whereas fraction 7 had CD value higher than the IC_{50}, and the fraction 8 had CD value close to the IC_{50} value (**Table 1**). The chromatographic profiles of active fractions were analyzed by reverse phase HPLC with a diode array detector to provide multiple wavelength analysis (not shown). Based on abundance and complexity of chemical profiles, fractions 3 and 8 were further resolved to obtain pure isolates. Compound **1** was afforded from fraction 3 by sequential purification using reverse phase flash chromatography, silica gel preparative TLC, and reverse phase preparative HPLC (**Figure 3**). Compound **2** was isolated from fraction 8 by reverse phase flash chromatography and preparative HPLC (**Figure 4**).

Fractionation of the EA-ME Isolate. Six fractions were generated from the EA-ME isolate using silica gel flash chromatography, and the bioassay revealed that the first three fractions had potent QR-inducing activities with CD values between 10 and 20 μg/mL (**Table 1**). Examination of HPLC chromatograms indicated that fractions 1 and 2 were complex of mixtures of compounds of similar abundance, which made prospects for further purification appear difficult. In contrast, fraction 3 exhibited two dominant peaks (data not shown), which was also confirmed by TLC analysis, as well as other minor and well-spaced peaks. Moreover, among these three active fractions, the fraction 3 was the most abundant, accounting for more than 70% of total weight of these three collective fractions. Further purification of fraction 3 using flash chromatography and preparative HPLC afforded compounds **3** (**Figure 5**), **4**, and **5** (**Figure 6**).

Identification of Isolated Pure Compounds. Compound **1** was obtained as a light brown oil and had a molecular formula of $C_{18}H_{18}O_5$ as established from its HREI and ^{13}C spectra. In the ^1H NMR spectrum, one set of ABX aromatic proton signals at δ 6.78 (*d*, $J = 8.0$ Hz, H-5), 7.10 (*d*, $J = 8.4$, 2.0 Hz, H-6), and 7.31 (*d*, $J = 2.0$ Hz, H-2) showed the presence of a 1,3,4-trisubstituted benzene moiety in the molecule (*31*). Two protons at δ 6.44 and 7.52 with a large coupling constant ($J = 16$ Hz) indicated *trans* olefinic protons (*32*). From HMBC and HMQC spectra, three protons of the methoxyl group at δ 3.81 correlated with C-3 (δ_C 147.9), and the *trans* olefinic proton at δ 7.52 correlated with carboxyl carbon (δ_C 166.5) and C-1 (δ_C 125.352), C-2 (δ_C 111.1), and C-6 (δ_C 123.2) of the 1,3,4-trisubstituted benzene moiety, indicative of the presence of a feruloyl moiety in the molecule. Two coupled triplet protons at δ 2.83 and 4.25 (each 2H, *d*, $J = 7.0$ Hz) and the correlation between one triplet proton (δ_H 4.25) and carboxyl carbon indicated the existence of the $-COO-CH_2-CH_2-$ structure (**Figure 2**). Downfield aromatic protons at δ 6.69 (2H, *d*, $J =$ 8.4 Hz, H-3′ and H-5′) and 7.07 (2H, *d*, $J = 8.4$ Hz, H-2′ and H-6′) and their corresponding carbons at δ 115.1 (2C, C-3′ and C-5′) and δ 129.6 (2C, C-2′ and C-6′) indicated a symmetrical 1,4-disubstituted benzene moiety. Existence of a *p*-hydroxyphenethyl moiety was deduced by correlations between methylene proton H-7′ (δ_H 2.83) and C-1′ (δ_C 127.9), C-2′ (δ_C 129.6), and C-6′ ($_C$ 129.6) in HMBC spectrum (**Figure 2**). Taken together, compound (**1**) was identified as *p*-hydroxyphenethyl *trans*-ferulate, and the NMR data were consistent with literature with minor differences conferred by different NMR solvents used (*33*). *p*-Hydroxyphenethyl *trans*-ferulate is a free radical scavenger and has been isolated from several botanical sources (*34*) including coriander (*Coriandrum sativum*) (*35*) and mallow (*Sida spinosa* L.) (*33*).

Compound (**2**) was obtained as a white amorphous solid and was identified as 5,6-dimethyl-2-pyridine carboxylic acid. Its EI and positive ESI mass spectra showed $[M]^+$ and $[M + H]^+$ signals at *m*/*z* 151 and 152, respectively. HREI analysis and the ^{13}C NMR spectrum disclosed the molecular formula to be $C_8H_9NO_2$. In the EI mass spectrum, the presence of signals at *m*/*z* 107 $[M + H - COOH]^+$ (100) and 106 $[M - COOH]^+$ (58) suggested a carboxylic acid group, which was consistent

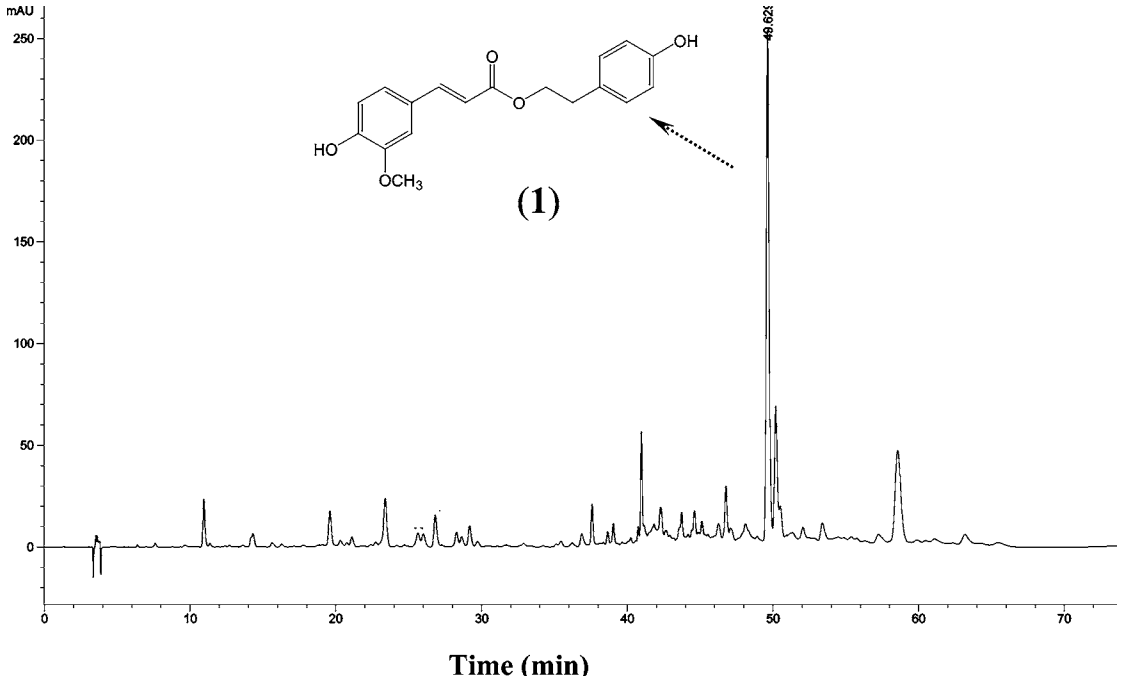

Figure 3. Reverse phase HPLC chromatogram of fraction 3 from HX-ME isolate.

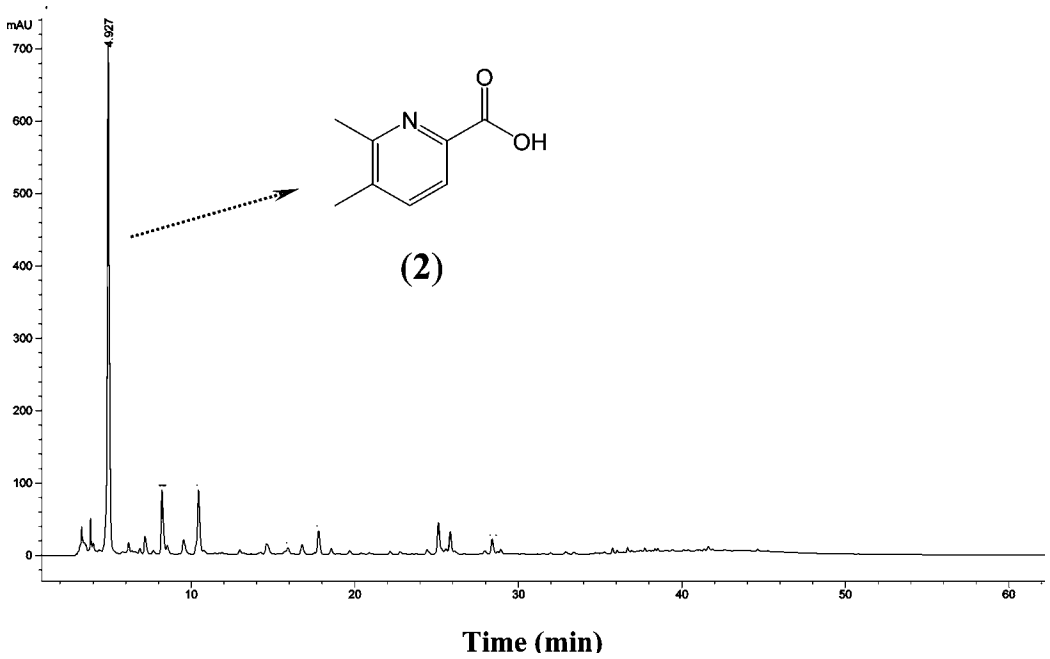

Figure 4. Reverse phase HPLC chromatogram of fraction 8 from HX-ME isolate.

with a downfield signal for carbonyl carbon (δ_C 164.5) in the ^{13}C spectrum. The presence of a pyridine moiety in the molecule was deduced from five downfield carbon signals and one nitrogen atom in the molecular formula. The long-range correlation (**Figure 2**) between one of two coupled aromatic protons, H-3 (δ_H 7.96), and the carboxyl carbon (δ_C 164.5) indicated that the two protons were in *ortho* and *meta* positions relative to the carboxyl group on the pyridine ring. In the HMBC spectrum, two sets of methyl protons (3 each, δ_H 2.38 and δ_H 2.55) correlated with both C-5 (δ_C 137.2) and C-6 (δ_C 156.3), providing evidence for the *ortho* relationship of the two methyl groups on the pyridine ring. No report was found in the literature to indicate any natural source of this compound. However, it has been used for the synthesis of 2-pyridinecarboxamide derivatives as antiallergic agents (*36*).

Compound **3** was identified as ferulic acid, and its NMR spectra were consistent with those of the authentic compound purchased commercially.

Compound **4** was obtained as a light brown amorphous solid, and a molecular formula of $C_8H_9NO_2$ was deduced from HREI and the ^{13}C spectrum. The high-field proton triplet (δ_H 1.06, 3H, J = 7.0 Hz) and quartet (δ_H 3.07, 2H, J = 7.5 Hz) and their long-range correlations with the ketone carbon (δ_C 200.2) suggested the presence of the $-C(O)CH_2CH_3$ structure (**Figure 2**). This was also confirmed by the EI mass spectrum with an ion signal at m/z 94 $[M - C(O)CH_2CH_3]^+$ (100). One set of ABX aromatic proton signals at δ 7.22 (1H, d, J = 8.5 Hz), 7.85 (1H, d, J = 8.5 Hz), and 8.16 (1H, s), and five downfield carbon signals indicated a disubstituted pyridine ring in the molecule. The HMBC spectrum provided the following cor-

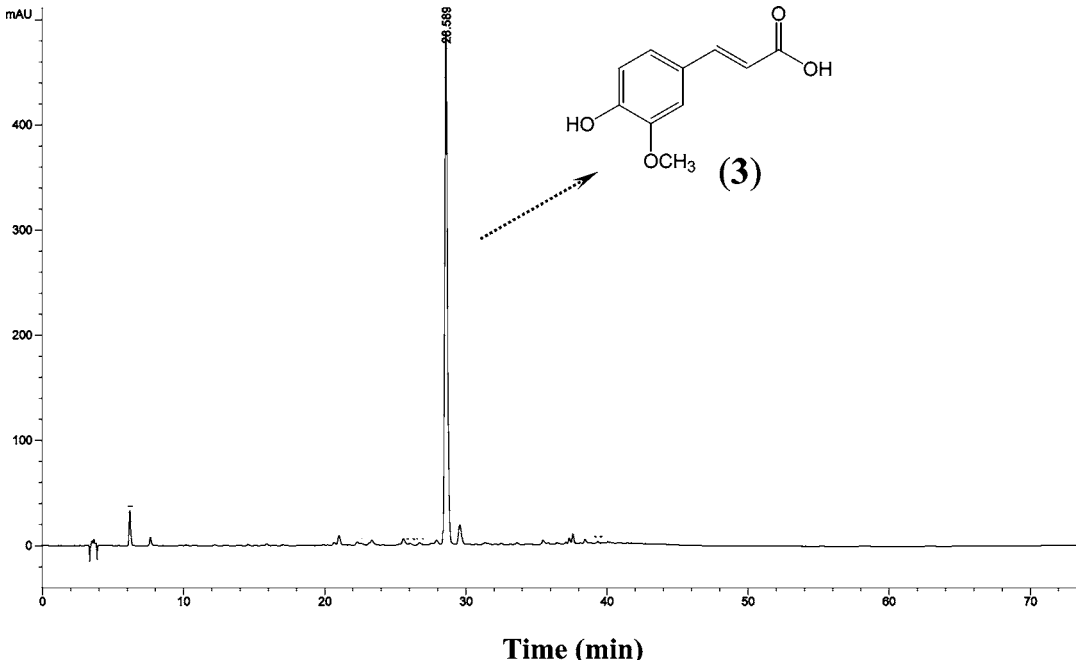

Figure 5. Reverse phase HPLC chromatogram of fraction 3B from HX-ME isolate.

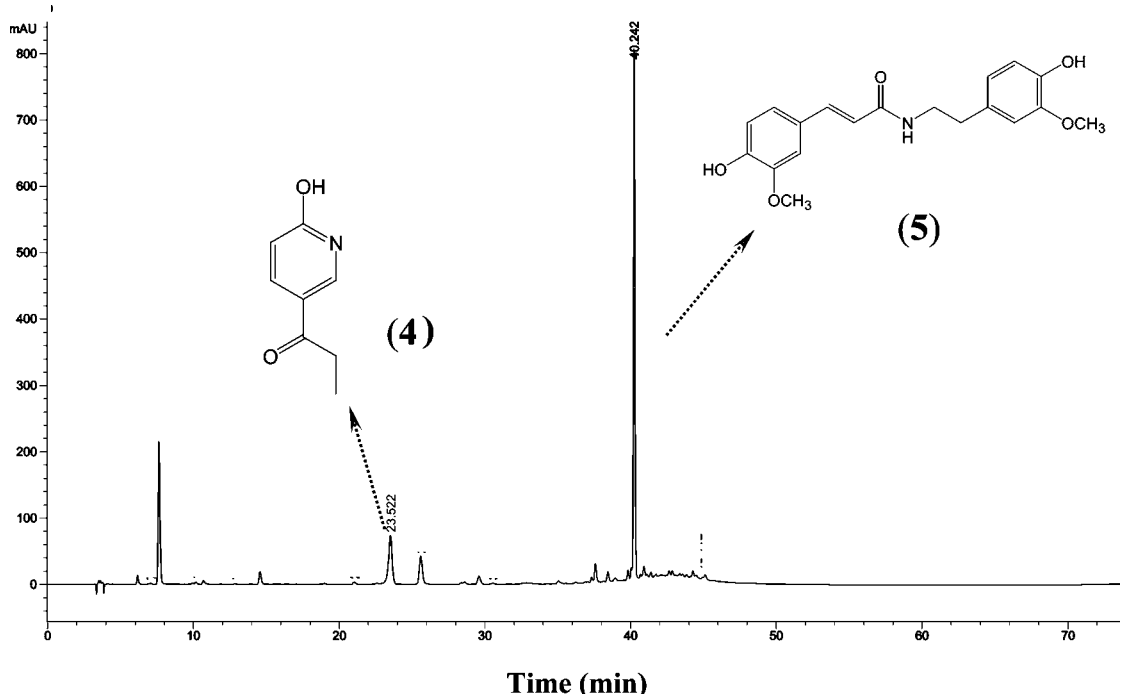

Figure 6. Reverse phase HPLC chromatogram of fraction 3C from HX-ME isolate.

relations: H-4 to C-6 and ketone carbon; H-2 to C-3, C-4, and C-6; and H-5 to C-3 (**Figure 3**). Collectively, these data indicate that compound **4** must be 1-(6-hydroxy-[3]pyridyl)-propan-1-one. To our knowledge, this compound has not been reported from any natural source, but it was synthesized from 1-(6-ethoxy-[3]pyridyl)-propan-1-one by hydrolysis with hydrogen chloride (Beilstein database, Beilstein chemical registry number 121959, reaction ID 125155).

Compound **5** was isolated as a light brown oil. Its HRESI spectrum showed the $[M + H]^+$ ion signal at *m*/*z* 344.1496, which suggested the molecular formula of $C_{19}H_{21}NO_5$ based on eighteen ^{13}C signals (one of the ^{13}C signals was from two carbons as indicated in the HMQC spectrum). There were two sets of ABX aromatic proton signals in the ^1H spectrum. One set of them, at δ 6.78 (*d*, $J = 8.4$ Hz), 6.97 (*dd*, $J = 8.4$, 1.6 Hz), and 7.10 (*d*, $J = 1.6$ Hz), and the existence of two protons at δ 6.43 and 7.30 with a large coupling constant ($J = 16$ Hz), were similar to those of compound **1**. Further analysis of the ^{13}C spectrum suggested compound (**5**) also had a feruloyl moiety. The second set of ABX proton signals at δ 6.60 (*dd*, $J = 8.0$, 1.6 Hz), 6.68 (*d*, $J = 7.6$ Hz), and 6.77 (*d*, $J = 1.6$ Hz) indicated another 1,3,4-trisubstituted benzene ring structure in the molecule [41]. A proton triplet at δ 7.97 ($J = 5.6$ Hz) was assigned as −NH− because no correlated carbon was found in the HMQC spectrum. From the HMBC spectrum, this −NH− proton had correlations with the carboxyl carbon (δ_C 165.2) and a methylene carbon C-8′ (δ_C 40.4), which indicated the structural unit of −C(O)−NH-CH_2CH_2− (**Figure 2**). The presence of

Table 2. Phase II Enzyme Induction by Pure Isolates[a]

			quinone reductase			glutathione S-transferase	
compound	dose range (μM)	IC_{50} (μM)	I_{max} (μM)	CI_{max} (μM/(μg/mL))	CD (μM/μg/mL)	I_{max} (μM)	CI_{max}
1	1–572	86 ± 1	3.64 ± 0.09	71.5/(22.5)	6.6 ± 0.9/2.1 ± 0.3	1.70 ± 0.08	*c*
2	13–6620	5080 ± 185	3.55 ± 0.56	3310/(500)	754 ± 66/114 ± 10	1.65 ± 0.16	*c*
3	5–2550	2310 ± 201	1.71 ± 0.004	1275/(248)	*b*	1.94 ± 0.19	*c*
4	13–6620	3860 ± 243	3.81 ± 0.46	3310/(500)	624 ± 75/94 ± 11	1.47 ± 0.09	*c*
5	2.5–1250	463 ± 63	3.74 ± 0.8	313/(107)	61 ± 17/21.0 ± 5.9	1.76 ± 0.25	*c*

[a] IC_{50}, concentration required to reduce cell protein to 50%; I_{max}, maximum induction ratio; CI_{max}, concentration for maximum induction; CD, concentration required for doubling enzyme specific activity; CI_{max} and CD were mean ± standard deviation of at least three replicates. All measured values reported in this table were significantly different ($p < 0.01$) from the controls by a two-tailed Student's *t*-test. [b] Not applicable; did not double QR specific activity. [c] Same as for QR.

methoxytyramine moiety was deduced from the following correlations: methylene proton H-8′ (δ_H 3.36) to C-1′ (δ_C 130.1) and methylene proton H-7′ (δ_H 2.65) to C-1′ (δ_C 130.1), C-2′ (δ_C 112.7), and C-6′ (δ_C 120.6). From all the above observations, compound **5** was identified as *N-trans*-feruloyl 3-*O*-methyldopamine. This compound has been previously isolated from various botanical sources (*34*) including spinach (*Spinacia oleracea*) leaves (*37*).

Phase II Enzyme-Inducing Activities of Pure Isolates. Pure compounds **1**−**5** were tested for inducing activity on QR and GST (**Table 2**). *p*-Hydroxyphenethyl *trans*-ferulate (**1**) had a CD value of 6.6 μM (2.1 μg/mL) for QR and was able to induce QR and GST activity up to 3.64- and 1.7-fold, respectively, compared with the control. 5,6-Dimethyl-2-pyridine carboxylic acid (**2**) and 1-(6-hydroxy-[3]pyridyl)-propan-1-one (**4**) induced QR (>3-fold) at high concentrations and had CD values greater that 500 μM. Ferulic acid (**3**) weakly induced both QR and GST activity and failed to double their activity at the concentrations up to the IC_{50} value of this compound (~2300 μg/mL). *N-trans*-Feruloyl 3-*O*-methyldopamine (**5**) induced QR up to 3.74-fold at a relatively high concentration (313 μM) with a CD value of 61 μM. Although the CD value of compound (**1**) was about one-third of that of the parent fraction (2.1 *versus* 6.2 μg/mL), fraction 3 of the HX-ME isolate, the natural abundance of this compound only accounted for about 1.2% of the total weight of fraction 3, which may suggest that there are other constituents having more potent inducing power in this parent fraction or acting synergistically with these pure isolates. Compounds **2**, **4**, and **5** had CD values greater than that of their parent subfractions, fraction 8 of HX-ME isolate and fraction 3 of the EA-ME isolate. This also indicated the possible presence of other more potent QR-inducers in these two fractions.

Compounds **1**, **3**, and **5** are coumaric (hydroxycinnamic) acid derivatives, and all have the α,β-unsaturated carbonyl functional groups characteristic of typical "Michael acceptors". Many phase II enzyme inducers possess Michael acceptor-type structures and their inducing potencies closely correlate with their Michael reactivities (*4*). Michael reactivity depends on the nature of electron-withdrawing groups conjugated with the olefin structure. In general, the reactivity decreases as electron-withdrawing group = COAr > CHO > $COCH_3$ > CO_2CH_3 > CN > $CONH_2$ ≫ COOH. Compound **1** has a $-CO_2CH_3$ electron-withdrawing group, while compound **5** has a $-CONH_2$ electron-withdrawing group, making it a weaker Michael acceptor and QR inducer than compound **1**. Since a free carboxylic acid is a very weak Michael acceptor, compound **3**, ferulic acid, did not double QR activity as has been reported previously (*38*).

In conclusion, nine partially purified isolates obtained from crude hexane and ethyl acetate extracts of green onion exhibited potent QR-inducing activity (CD values <20 μg/mL) in Hepa 1c1c7 cells. Further fractionation of selected active fractions afforded five pure compounds, and their inducing activities were evaluated. All pure compounds were confirmed to induce QR in Hepa 1c1c7 cells, and they differed in terms of potency and maximum extent of induction. The purified compounds also contained α,β-unsaturated carbonyl structural units, consistent with the properties of many QR inducers to function as Michael reaction acceptors (*4*). *p*-Hydroxyphenethyl *trans*-ferulate (**1**) showed strong QR-inducing activities with CD values of 6.6 μM (2.1 μg/mL), while the structurally related *N-trans*-feruloyl 3-*O*-methyldopamine (**5**) exhibited the next most potent QR-inducing activity with a CD value of of 61 μM (21 μg/mL). Both of these compounds are known to be present in other botanical species and may be widespread among edible plants. To our knowledge, 5,6-dimethyl-2-pyridinecarboxylic acid (**2**) and 1-(6-hydroxy-[3]pyridyl)-propan-1-one (**4**) have not previously been isolated from a natural source. The origin of these pyridine derivatives is unknown, but they may possibly be formed by the conditions prevailing during solvent extraction, namely, refluxing temperatures of hexane and ethyl acetate and relative lack of water.

Results also indicated that other unidentified agents, with potentially stronger inducing power or acting synergistically with the isolated pure compounds, may exist in the isolated active fractions. This warrants continued efforts to isolate and identify other QR-inducing agents from green onion extracts. The QR-inducing activity of isolates from green onion is consistent with the view that consumption of fruits and vegetables, and especially Alliums, may reduce risk of cancer in humans (*39*, *40*). Identifying onion compounds that are also efficacious *in vivo* may help identify or develop strains that are enriched in such compounds for the purpose of delivering cancer chemopreventive benefits through dietary interventions.

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