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Phenolic Derivatives from Soy Flour Ethanol Extract Are Potent In Vitro Quinone Reductase (QR) Inducing Agents

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The fractionation of soy flour directed by a cellular bioassay for induction of phase 2 detoxification enzymes was used to identify quinone reductase (QR) inducing agents. A phospholipid-depleted, 80% methanol-partitioned isolate from a crude ethanol extract of soy flour was resolved using normal phase medium-pressure liquid chromatography (MPLC). Early eluting fractions were found to be the most potent QR inducing agents among the separated fractions. Fraction 2 was the most potent, doubling QR at <2 μg/mL. Further fractionation of this isolate led to the identification of several constituents. Fatty acids and *sn*-1 and *sn*-2 monoacylglycerols were identified, but were not highly potent QR inducers. Benzofuran-3-carbaldehyde, 4-hydroxybenzaldeyde, 4-ethoxybenzoic acid, 4-ethoxycinnamic acid, benzofuran-2-carboxylic ethyl ester, and ferulic acid ethyl ester (FAEE) were also identified as QR inducing constituents of this fraction. FAEE was the most potent of the identified constituents, doubling QR specific activity at 3.2 μM in the cellular bioassay.

KEYWORDS: Soy; soy flour; quinone reductase; QR; Hepa 1c1c7; ferulic acid ethyl ester; benzofuran; fatty acid; monoacylglycerol; phenolic derivatives

INTRODUCTION

Induction of phase 2 detoxification enzymes by dietary components is a potential cancer chemopreventive strategy (*1*). Plants are abundant sources of phytochemicals that can induce phase 2 enzyme metabolism of xenobiotics (*2*). In the interest of screening for agents that promote phase 2 enzyme induction, rapid screening methods have been developed for quinone reductase (QR) activity in the murine liver Hepa 1c1c7 cell line (*3*). QR catalyzes the two-electron reduction of quinones to hydroquinones and is coordinately induced with other phase 2 detoxification enzymes, such as UDP-glucuronosyltransferase and glutathione-*S*-transferase (*4*). QR inducers have structural elements that are electrophilic, reactive with sulfhydryl groups, glutathione-depleting agents, or indirectly involved in the alteration of redox status (*2*). Consumption of foods and/or supplements enriched in phase II enzyme inducers has the potential to reduce cancer risk in humans. Previous studies have identified extracts from green onion, broccoli, Brussels sprouts, and ginger, among others, as potent phase 2 enzyme inducers in the Hepa 1c1c7 cell line (*5*). Dietary supplementation of sulforaphane, quercetin, and curcumin also increases activity of phase 2 enzymes in rodents (*6, 7*).

Soy has been investigated extensively for its prospective cancer preventive potential (*8*). Phytoestrogens, sterols, phytic acid, protease inhibitors, and phenolics from soy are considered to be potential cancer chemopreventive agents (*9*−*11*). Soy flour and protein isolates are known to induce phase 2 enzymes in rodents (*12*). However, the phytochemicals directly responsible for this effect have not been identified.

Soy isoflavones have been observed to induce the phase 2 enzyme marker QR in the murine Hepa 1c1c7 line (*13*). Bioactivity-directed fractionation of an ethanol extract of soy flour demonstrated isoflavones were abundant but only partially contributed to in vitro QR induction by the isolates obtained (*14*). In vivo, dietary administration of isoflavones is not broadly effective at inducing phase 2 enzymes. A hydrolyzed isoflavone fraction was found to induce QR in mouse lung, but did not in the liver, kidney, stomach, or small intestine (*15*). Dietary supplementation of genistein in rats does not induce hepatic QR (*16, 17*). Therefore, it is hypothesized that other in vivo QR inducing agents exist in soy. The aim of this study was to purify and characterize in vitro QR inducers from an ethanol extract of soy flour.

MATERIALS AND METHODS

Chemicals and Supplies. Reagents and chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). HPLC-grade solvents were acquired from Fisher Scientific (Chicago, IL). Costar 96-well microtiter plates were obtained from Corning Inc. (Corning, NY). Cell culture medium and supplements were from Invitrogen

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(Carlsbad, CA). Soy flour was freshly milled food-grade product from Didion Mills in Johnson Creek, WI. Flour was stored in sealed bags at −20 °C in darkness until use.

Enrichment of QR-Inducing Activity in an Ethanol Extract from Soy Flour. An ethanol extract further enriched in QR-inducing activity was obtained as an 80% methanol isolate as described in a previous study (*14*) and as outlined in the Supporting Information. Briefly, ~900 g of soy flour was successively extracted by ethyl acetate and then by ethanol under reflux conditions using large Soxhlet extraction glassware. Ethanol solubles were partitioned between silica gel and hexane to remove phospholipids and further partitioned between 80% methanol and hexane. The 80% methanol extracted solids doubled QR specific activity at 42 μg/mL and were selected for further fractionation and identification.

Medium-Pressure Liquid Chromatography (MPLC). The 80% methanol isolate was fractionated using normal phase medium-pressure liquid chromatography (MPLC). An aliquot (5 g) of the 80% methanol isolate was dissolved in 25 mL of methanol and mixed with 15 g of silica gel (60 Å, 230−400 mesh, Fisher Scientific, Fair Lawn, NJ). Solvent was removed from this mixture using rotary evaporation to yield a free-flowing powder, which was loaded into a 4.8 cm × 10 cm sample precolumn, coupled to a 4.8 cm × 25 cm separating column packed with 250 g of silica gel and preconditioned with methylene chloride. Elution of components from the column was controlled by a peristaltic pump using a stepwise gradient of 0, 2.5, 5, 10, 20, 50, and 100% methanol in methylene chloride, in 500 mL increments at a flow rate of 15 mL/min. Fractions of eluate were collected in 50 mL glass tubes every 3 min with a Foxy Jr. fraction collector (Isco Inc., Lincoln, NE). Aliquots (150 μL) from each tube were transferred to a quartz 96-well microtiter plate and scanned at six different wavelengths (210, 230, 250, 280, 330, 450 nm) using an optical plate reader (SpectraMax Plus, Molecular Devices, Sunnyvale, CA). Fractions were pooled according to the apparent segregation of material absorbing at 280 nm. Dry matter was recovered from pooled fractions by vacuum rotary evaporation at 40 °C. Dry matter from each fraction was transferred to glass test tubes after dissolving in 20% methanol in methylene chloride and subsequently recovered by evaporation of solvent under a nitrogen gas stream in a 40 °C water bath. Isolates were stored at −20 °C in darkness until use.

Thin Layer Chromatography (TLC) Analysis. Isolates were further separated by preparative TLC. Samples were spotted on 20 × 20 cm silica plates (PK6F silica gel 60 Å, 1000 μm, Whatman, Inc., Florham Park, NJ) and developed in 7 or 20% methanol in methylene chloride (v/v). Resolution of samples on plates was visualized under UV light at 254 and 365 nm and facilitated by H_2SO_4 charring of a narrow strip of the TLC plate.

High-Pressure Liquid Chromatography (HPLC). Reverse phase analytical HPLC was used to screen MPLC fractions for complexity and to develop appropriate preparative HPLC methods. For analytical HPLC, an Agilent 1100 HPLC was equipped with a degasser, quaternary pump, autosampler, and diode array detector (Agilent Technologies, Inc., Santa Clara, CA). For preparative HPLC, two ISCO model 2350 HPLC units with a UV detector (V^4, ISCO Inc.) and integrator (Spectra-Physics model 2350, San Jose, CA) were used to monitor eluates at 280 nm. Binary mixtures of acetonitrile or methanol in water with 2% acetic acid were used as mobile phases. Discovery C-18 columns (Supelco, Bellefonte, PA) were used for preparative (25 cm × 21.2 mm, 5 μm), semipreparative (25 cm × 10 mm, 5 μm), and analytical (25 cm × 4.6 mm × 5 μm) methods.

Gas Chromatography−Mass Spectrometry (GC-MS) Analysis. Chemical constituents of oily fractions were identified by GC-MS and GC was used for quantitative analysis of these constituents. Lipids were derivatized using *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TCMS) kits from Supelco. BSTFA (200 μL) and TCMS (100 μL) were added to 0.1 mL of an oily fraction or standards dissolved in pyridine and heated for 60 min at 70 °C. Tetradecane (100 mg/25 mL pyridine or hexane) was used as an internal standard. Equal volumes of hexane and water were added to the reaction mixture to partition the lipids into the hexane phase. An Agilent 6890 GC with a flame ionization detector (FID), autoinjector, and a 30 m × 0.250 mm DB-17 ms column (J&W Scientific, Folsom, CA) was used to resolve the mixture. A published method was used to separate typical vegetable oil monoacylglycerols and diacylglycerols from a 1 μL sample injection of the hexane phase (*18*).

For GC-MS, an Agilent 6890 with a 5973 MSD and FID with the same column, injection parameters, and temperature profile was run in splitless mode. Source temperature was 230 °C with the quadrupole set at 150 °C Electron ionization at 70 eV was applied during the scanning of a mass range from *m*/*z* 50 to 550. Samples were compared to the software library (NIST version 1.7 spectral database, Agilent) and with authentic standards for identification.

Nuclear Magnetic Resonance. NMR was performed at the Analytical Instrumentation Center (AIC) at the University of Wisconsin−Madison School of Pharmacy. A Varian UI-500 (Varian, Inc., Palo Alto, CA) spectrometer was equipped with either a Varian hcx4765 probe for proton and indirect detection experiments, a Nalorac qn6121 probe for ^{13}C experiments, or a Protasis icg1032 capillary probe (Protasis/MRM, Savoy, IL) for ^1H and indirect detection experiments with mass-limited samples. Solvent-matched 5 mm glass tubes were routinely used for analysis (Shigemi, Inc., Allison Park, PA).

Mass Spectrometry Analysis. Mass spectrometry of purified components was also performed at the campus AIC. Atmospheric pressure chemical ionization (APCI) was performed on an Agilent 1100 LC-MSD quadrupole instrument. An isocratic gradient of methanol (80%)/water (20%) was used to resolve appropriately diluted samples (10 μL) autoinjected inline onto a C-18 guard column (Waters Corp., Milford, MA). Electrospray ionization (ESI) was performed using an Agilent 1100 MSD Trap SL instrument, using direct infusion. MS^n experiments used nitrogen gas for chemical-induced collision (CID).

Quinone Reductase (QR) Bioassay. The Prochaska method was used to assay QR induction in murine Hepa 1c1c7 cells (ATCC, Manassas, VA) (*3*). Cells were grown in α-minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and antibiotic (Invitrogen). For QR induction experiments, cells were seeded at 5000 cells/well in duplicate plates and incubated at 37 °C in a 5% CO_2 atmosphere for 24 h. Medium was then decanted, and test isolates dissolved in growth medium containing 0.1% hydroxypropyl β-cyclodextrin (HPBCD) as a carrying agent were added to the wells in half-step dilutions. Cells were incubated for an additional 48 h. For QR analysis, a digitonin−EDTA solution was added prior to the initiation of a menadione-linked NADPH-dependent reduction of MTT dye monitored for 10 min at 490 nm using an optical plate reader. The duplicate plate for protein analysis was decanted of culture medium, soaked in a crystal-violet containing solution for 10 min, rinsed and soaked with water, decanted, and held in a 95% ethanol solution with 1% SDS for 60 min before reading at 690 nm in the optical plate reader.

Absorbance readings for background wells were subtracted from cell responses, which were then normalized to control cells (no test compound added) for QR and protein readings. Relative specific activity was calculated as the following ratio: $(QR/protein)_{test\ isolate}/(QR/protein)_{control}$. Concentrations required to double QR specific activity (CD value) and causing a reduction in protein of ≥50% (IC_{50} values) were interpolated from a semilog plot of the corresponding data. A chemopreventive index (CI) was calculated as the ratio of the IC_{50}/CD values (*19*). As a measure of potency, inducing units (IUs) were calculated as (*3*):

$$\text{IUs/g} = \frac{10^6\ \mu\text{g/g}}{(\text{CD value in } \mu\text{g/mL}) \times 0.15\ \text{mL of cell culture medium}}$$

Statistical Analysis. Significance testing for QR specific activity was done by comparing isolates to the control using a two-tailed *t* test. Significance was defined at a *P* value of <0.01, as this was a prospective study. CD value variance was defined as the average CV of QR specific activity measurements at levels of isolate immediately adjacent to the CD value.

RESULTS

MPLC Fractionation. Stepwise elution of the 80% methanol isolate using normal phase silica chromatography was collected

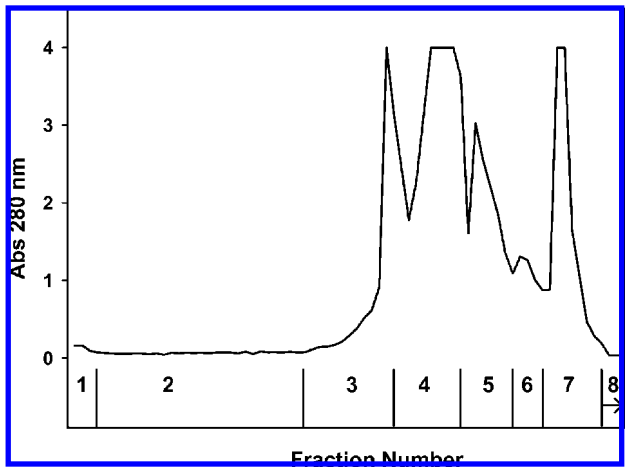

Figure 1. Normal phase medium-pressure liquid chromatography profile of fractions obtained from an 80% methanol-partitioned isolate from an ethanol extract of soy flour. Numbers indicate arbitrary designation of fractions based on absorbance of eluate at 280 nm.

Table 1. Recoveries and QR Inducing Potency of MPLC Fractions from 5 g of an 80% Methanol-Partitioned Isolate from an Ethanol Extract of Soy Flour

fraction	recovery (g)	% recovery (mass basis)	CD value[a] (μg/mL)	CD value CV[b]
1	0.0064	0.13	13	12
2	0.0106	0.22	<2	–[c]
3	0.0658	1.3	32[d]	18
4	0.1717	3.4	43	13
5	1.9656	39	601	27
6	1.3592	27	>1000	–
7	1.5322	31	>2000	–
8	0.0236	0.46	330	9

[a] CD values are the mean values of duplicate determinations. [b] CV is the average CV of QR specific activity measurements at tested levels of isolate immediately adjacent to the CD value. [c] –, not applicable. [d] Fraction 3 also doubled QR specific activity in the range of 2.0–7.8 μg/mL (cf. **Figure 2**), but doubling was not maintained at increasing levels until 32 μg/mL was reached.

as eight fractions as guided by absorbance profiles at 280 nm (**Figure 1**). Fractions 1–4 contained 5% of the recovered mass and were the most potent of the resolved isolates (**Table 1**). Of these fractions, fraction 2 was the most potent QR inducing isolate, with a CD value of <2 μg/mL (**Figure 2**). Fractions 1, 3, and 4 doubled QR in the range of 31–44 μg/mL (**Table 1**). The bulk of the remaining mass was recovered in fractions 5–7, and these fractions were at least 10-fold less potent than fractions 1–4. Fraction 2 was further analyzed on the basis of the high potency recovered in this isolate.

Characterization of Fraction 2. MPLC fraction 2 was pooled from two column runs, which accounted for 35 mg of the 13 g of the loaded dry matter. Preparative TLC yielded distinct bands with R_f values of 0.9, 0.8, 0.7, and 0.5, designated bands 1–4, respectively. Bands were scraped, eluted with 20% methanol in methylene chloride, and then further analyzed by GC-MS (band 1) or separated using preparative HPLC (bands 2–4). Band 4 was the most potent QR inducing fraction, with a CD value of 1.3 μg/mL (**Table 2**). Band 1 had a CD value of 11 μg/mL, the least IUs/g (6.1×10^5), and least total IUs (7900), a measure of total recovered QR inducing potency. Bands 2 and 3 accounted for 40% of the recoverable IUs from the silica column and had intermediate CD values compared to bands 1 and 4.

TLC Band 1. The dry matter recovered in TLC band 1 was oily. Proton NMR of this fraction yielded a spectrum charac-

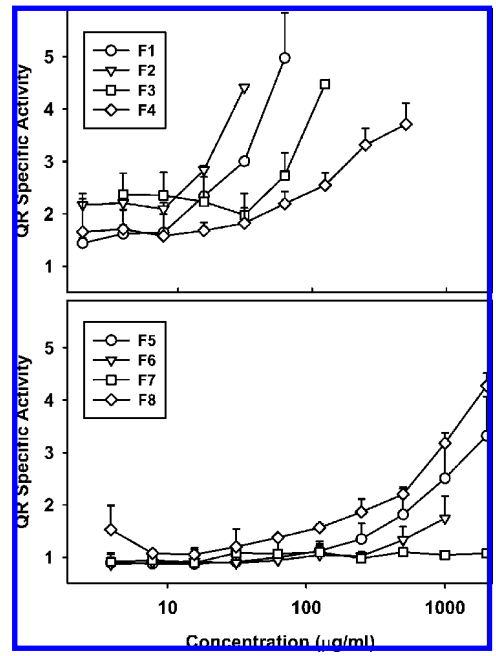

Figure 2. Quinone reductase induction in murine Hepa 1c1c7 cells by normal phase medium-pressure chromatography fractions (F1–8) prepared from an 80% methanol-partitioned isolate from an ethanolic extract from soy flour. Results reflect mean values of duplicate determinations.

Table 2. Recovery and QR Inducing Potency of Preparative TLC Isolates Obtained from MPLC Fraction 2

TLC band	R_f	recovery (mg)	CD value[a] (μg/mL)	CV[b]	IUs [c]/g	total IUs	recovered IUs % of fraction 2
1	0.9	13	11	44	6.1×10^5	7900	16
2	0.8	4.9	3.3	14	20.0×10^5	9900	21
3	0.7	3.4	2.5	16	27.0×10^5	9100	19
4	0.5	4.1	1.3	17	51.0×10^5	21000	44

[a] CD values are mean values of duplicate determinations. [b] CV is the average CV of QR specific activity measurements used to determine the CD value. [c] IUs (inducing units) is a measure of recovered QR inducing potency, where IUs = mass of fraction (g) $\times 10^6$/(CD-value $\times$ 0.15), where 0.15 is the volume (mL) of medium used in the enzyme assay (*5*).

Table 3. Mean Percent Composition and Standard Deviation ($n = 3$) of Fatty Acids (FA) and Monoacylglycerols (MAG) in TLC Isolated Band 1 of MPLC Fraction 2

lipid species	mean %
palmitic FA	0.39 ± 0.02
stearic FA + oleic FA	0.62 ± 0.09
linoleic FA	3.2 ± 0.4
linolenic FA	0.10 ± 0.02
palmitic *sn*-2 MAG	1.2 ± 0.0
palmitic *sn*-1 MAG	11 ± 0
stearic *sn*-2 MAG	0.63 ± 0.03
stearic *sn*-1 MAG + oleic *sn*-1 MAG	8.2 ± 0.1
oleic *sn*-2 MAG	1.4 ± 0.1
linoleic *sn*-1 MAG	39 ± 1
linolenic *sn*-1 MAG	21 ± 2

teristic of fatty acids and monoacylglyerols (*20*). GC-MS of this silated isolate led to the identification of fatty acids and monoacylglycerols common to soybean oil (**Table 3**). The identified lipids accounted for 86% of the mass from this fraction. Linoleic acid doubled QR specific activity at 120 μM, but the *sn*-1 monoacylglycerol could not double QR specific activity before reaching the IC_{50} value (**Figure 3**). Palmitic, stearic, oleic, and linolenic acids and *sn*-1 monoacylglycerols

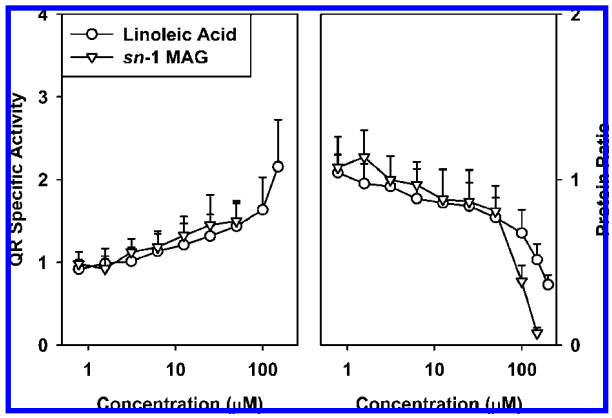

Figure 3. QR specific activity and protein ratio of Hepa 1c1c7 cells treated with linoleic acid and *sn*-1 linoleic monoacylglycerol (MAG). $n = 4$.

Table 4. Recovery and QR Inducing Potency of Preparative HPLC Fractions of TLC Isolates Obtained from MPLC Fraction 2

fraction							
TLC band	HPLC peak: identification	recovery (mg)	CD value[a] (μg/mL)	CV[b]	IUs/g[c] × 10^5	total IUs	recovered IUs
2	1	<0.1	ND	−[e]	ND	ND	ND
	2	0.2	49	15	1.4	27	2
	3	0.3	15	9	4.4	130	9
	4	0.2	14	17	4.8	95	7
	5	ND[d]	ND	−	ND	ND	ND
	6	ND	ND	−	ND	ND	ND
	7 [**1**]	0.3	4.5	12	15.0	440	31
	8	0.2	25	33	2.7	53	4
	9	0.2	7.0	18	9.5	190	13
	10	0.1	6.3	16	11	110	7
	11	0.1	1.7	19	39	390	27
3	1	<0.1	ND	−	ND	ND	ND
	2	0.1	30	17	2.2	22	9
	3	0.3	49	14	1.4	41	17
	4	0.2	18	4	3.7	74	31
	5	0.3	24	8	2.8	83	35
	6	<0.1	ND	−	ND	ND	ND
	7	<0.1	ND	−	ND	ND	ND
	8 [**2**]	0.4	160	22	0.4	17	7
	9	<0.1	ND	−	ND	ND	ND
	10	<0.1	ND	−	ND	ND	ND
4	1 [**3**]	1.3	21	18	3.2	410	9
	2 [**4**]	1.3	>40	−	<1.7	220	<5
	3 [**5**]	1.3	53	10	1.3	160	3
	4	1.5	4.7	8	14	2100	45
	5 [**6**]	1.7	6.3	16	11	1800	38

[a] CD values (concentration required to double QR activity) are mean values of two determinations. [b] CV is the average CV of QR specific activity measurements used to determine the CD value. [c] IUs (inducing units) are defined in **Table 2**. [d] ND, not determined due to insufficient dry matter recovered. [e] −, not applicable.

were tested for QR induction using the bioassay, and all exhibited similar or less QR inducing potencies relative to linoleic acid and linoleic *sn*-1 monoacylglycerol. Saturation, chain length, and *sn*-acyl chain position did not appear to affect the QR induction trend. Doubling of the QR specific activity correlated highly with a reduction in cellular protein levels, reflecting a loss in cell viability.

TLC Band 2. Preparative HPLC yielded 11 distinguishable absorbance peaks at 280 nm from TLC band 2, where peaks 1, 5, and 6 did not yield enough mass to test in the bioassay (**Table 4**). Peaks 7 and 9−11 had CD values of <10 μg/mL. Peaks 7 and 11 were the most potent and accounted for 58% of the recovered IUs from band 2. Peak 7 was identified as ferulic acid ethyl ester (FAEE) [**1**] and had a purity of 75% as estimated by NMR. FAEE was commercially available and had a CD value of 3.2 μM (0.7 μg/mL) with a CI of 19.

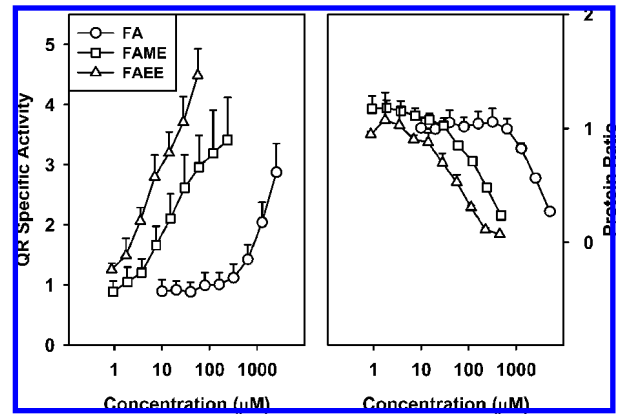

Figure 4. QR induction and protein ratio (a measure of cellular viability) in Hepa 1c1c7 cells by ferulic acid (FA), ferulic acid methyl ester (FAME), and ferulic acid ethyl ester (FAEE). $n = 8$.

TLC Band 3. Band 3 yielded 10 peaks at 280 nm using preparative HPLC. Dry matter recovered from peaks 1, 6, 7, 9, and 10 was insufficient to permit assays for QR induction (**Table 4**). None of the recovered HPLC fractions that could be assayed were as potent QR inducers as the parent fraction. Peak 8 was the most abundant fraction recovered from TLC band 3 and had a CD value of 160 μg/mL. This isolate comprised 7% of the recovered IUs and was identified as benzofuran-2-carboxylic acid ethyl ester [**2**] in 86% purity.

TLC Band 4. Band 4 was resolved into five peaks at 280 nm using preparative HPLC. Peaks 4 and 5 were the most potent and accounted for 83% of the recovered IUs (**Table 4**). Peak 1 had a CD value of 21 μg/mL and was identified as 4-hydroxybenzaldehyde [**3**] in 77% purity. The commercially available standard did not double QR specific activity up to 100 μg/mL, the highest concentration tested. This result may reflect QR inducing impurities copurified with peak 1. Peak 2 did not double QR specific activity up to the highest concentration tested of 40 μg/mL and was identified as benzofuran-3-carbaldehyde [**4**], in 93% purity. A standard was not available for comparison in the bioassay. Peak 3 was identified as 4-hydroxybenzoic acid ethyl ester [**5**], in 98% purity and had a CD value of 53 μg/mL. The commercial standard was also tested for QR induction and had a CD value of 90 μg/mL. Peak 4 could not be identified, but was the most potent with a CD value of 4.7 μg/mL. The MS APCI spectrum indicated an *m*/*z* of 206 as a possible molecular weight, with a water loss occurring in APCI (+) mode. However, NMR spectra revealed the presence of several impurities, and as a result of low abundance, resolution of connectivity was not achieved. The most abundant peak, peak 5, was identified as 4-ethoxycinnamic acid [**6**], in 89% purity. Peak 5 had a CD value of 6.3 μg/mL, whereas the commercially available standard had a CD value of 23 μg/mL.

Testing of Ferulic Acid Derivatives. Because ferulic acid is an abundant plant hydroxycinnamate, standard esters with increasing alkyl chain length were assayed for QR induction, including ferulic acid (FA), ferulic acid methyl ester (FAME), and FAEE. Potency corresponded with alkyl chain length substitution in the order of decreasing CD values of FA (1200 μM) < FAME (15 μM) < FAEE (3.2 μM) (**Figure 4**).

Spectral Data of Identified Components.

[1] Ferulic acid ethyl ester (TLC band 2, peak 7): $C_{12}H_{14}O_4$; MW 222.24; CAS Registry No. 4046-020-0; APCI (−), *m*/*z* 221.1 (100), 206.1 (32), 222.1 (15); ^{1}H NMR (CD_3OD,

500 MHz), δ 7.63 (1H, d, J = 15.9 Hz, H-7), 7.20 (1H, d, J = 1.8 Hz, H-2), 7.09 (1H, dd, J = 8.1, 1.8 Hz, H-6), 6.82 (1H, d, J = 8.1 Hz, H-5), 6.36 (1H, d, J = 15.8 Hz, H-8), 4.25 (2H, q, J = 7.1 Hz, OCH_2CH_3), 3.91 (3H, s, OCH_3), 1.34 (3H, t, J = 7.3 Hz, OCH_2CH_3). NMR and mass spectra agreed closely with the standard compound and ref *21*.

[2] Benzofuran-2-carboxylic acid ethyl ester (TLC band 3, peak 8): $C_{11}H_{10}O_3$; MW 190.20; CAS Registry No. 3199-61-9; APCI (−), *m/z* 188.2 (100), 189.2 (11); ^{1}H NMR (CD_3OD, 500 MHz), δ 8.08 (1H, d, J = 6.8 Hz, H-4), 7.98 (1H, s, H-2), 7.47 (1H, d, J = 7.3 Hz, H-7), 7.22 (2H, dt, J = 5.9 and 1.8 Hz, H-5/6), 4.38 (2H, q, J = 7.1 Hz, OCH_2CH_3), 1.44 (3H, t, J = 7.1 Hz, OCH_2CH_3). NMR and mass spectra agreed with those previously reported (*22*).

[3] 4-Hydroxybenzaldehyde (TLC band 4, peak 1): $C_7H_6O_2$; MW 122.12; CAS Registry No. 123-08-0; mass data, APCI (−), *m/z* 121.1 (100), 122.1 (8); ESI (+), *m/z* 122.80 (100); ^{1}H NMR (CD_3OD, 500 MHz), δ 9.69 (1H, s, CHO), 7.74 (2H, d, J = 8.6 Hz, H-2 and H-6), 6.84 (2H, d, J = 8.6 Hz, H-3 and H-5). MS of *m/z* (−) 121 agreed with APCI published spectra (*23*). NOESY analysis demonstrated through-space correlation between H-2/6 and H-3/5 and also between H-2/6 and H-1. gDQCOSY analysis demonstrated J-coupling between H-2/6 and H-3/5. Acetic acid ^{1}H δ 1.93, octadecylsilane δ 1.32, and an unknown impurity at δ 3.68 (0.5 H, s, HMBC δ 160.0) were detected in this fraction. NMR assignments closely matched published spectra (*24*).

[4] Benzofuran-3-carbaldehyde (TLC band 4, peak 2): $C_9H_6O_2$; MW 146.15; CAS Registry No. 4687-25-6; ESI-MS (+), *m/z* 145.95 (100); APCI (−), *m/z* 144.1 (100); APCI (+), *m/z* 145.1 (100), 118.1 (47) 147.1 (12); NMR ^{1}H NMR (DMSO-*d*, 500 MHz), δ 9.96 (1H, s, CHO), 8.27 (1H, s, H-2), 8.09 (1H, d, J = 7.8 Hz, H-3), 7.49 (1H, d, J = 7.8 Hz, H-6), 7.24 (2H, dt, J = 21.2 Hz-d, 7.3 Hz-t, H-4 and H-5). LC/APCI-MS peaks were evident at 0.241 min (APCI positive mode) and 0.271 min (APCI negative mode). ESI also showed *m/z* 104 (63), 187.0 (34), and 279.9 (24). The EI mass spectrum and NMR spectra agreed with published reference spectra (*25, 26*). gDQCOSY analysis revealed the following correlations: H-3 with H-4, H-4 with H-5, and H-5 with H-6. Additionally, TOCSY analysis revealed correlations of H-2 with H-3/4/6, H-3 with H-4, and H-6 with H-3/4.

[5] 4-Hydroxybenzoic acid ethyl ester (TLC band 4, peak 3): $C_9H_{10}O_3$; MW 166.18; CAS Registry No. 120-47-8; APCI (−), *m/z* 165 (100); ^{1}H NMR (CD_3OD, 500 MHz), δ 7.89 (2H, d, J = 8.8 Hz, H-2/6), 6.84 (2H, d, J = 8.7 Hz, H-3/5), 4.33 (2H, q, J = 7.3 Hz, OCH_2CH_3), 1.39 (3H, t, J = 7.36 Hz, OCH_2CH_3). The NMR spectrum matched closely with the standard compound. COSY interactions showed the presence of an ethyl group. HMBC and proton analyses also supported the location of the substitutions. The absence of a NOESY correlation from the ethyl group to a ring placed this group as an ethyl ester of the carboxylic acid. Furthermore, gHMBC revealed a correlation of the carbonyl with OCH_2CH_3 and ring H-2/6 protons. Silica and acetic acid were detected as impurities in the isolate.

[6] 4-Ethoxycinnamic acid (TLC band 4, peak 5): $C_{11}H_{12}O_3$; MW 192.21; CAS Registry No. 7362-39-2; ESI-MS (+), *m/z* 193.00 (100); APCI (−), 191.1 (100); NMR ^{1}H (CD_3OD, 500 MHz), δ 7.61 (1H, d, J = 16.1 Hz, H-7), 7.43 (2H, d, J = 8.6 Hz, H-2), 6.78 (2H, d, J = 8.4 Hz, H-2/6), 6.30 (1H, d, J = 15.9 Hz, H-3/5), 4.20 (2H, dd, J = 7.1 Hz, OCH_2CH_3), 1.28 (3H, t, J = 7.1 Hz, OCH_2CH_3). This NMR profile matched that of the standard compound, and the isolated material was a white solid.

DISCUSSION

QR inducing potency was highest in the most nonpolar, early eluting fractions obtained using normal phase MPLC. Fractions 1 and 2 had limited absorptivity at 280 nm compared to later eluting fractions. Lipids have poor UV absorbance at 280 nm, and the physical (oily) nature of fraction 1 indicated that a significant portion of it was lipid. Fraction 2 was derived from a large volume of pooled eluting material that would be considered the baseline of the chromatogram, with little absorptivity at 280 nm. This fraction had a complex fraction profile as judged by analytical HPLC, but because it was the most potent of the fractions eluted using MPLC, efforts were made to further resolve and characterize the chemical nature of this fraction. Preparative TLC was chosen as an intermediate means to further resolve MPLC fraction 2.

The lipids in TLC band 1 from fraction 2 were common to those observed in soybean oil (*27*). QR induction by lipid standards was weak, although doubling was observed near the IC_{50} value for linoleic acid (**Figure 3**). Palmitic acid, stearic acid, oleic acid, and linolenic acid conferred similar cellular responses. Monoacylglycerols at similar concentrations generally did not induce QR specific activity at levels where <50% loss of viability occurred, except for linolenic *sn*-1 MAG (**Figure 3**). Because none of the lipids identified in this fraction were as potent as the parent fraction 2, the compounds responsible for QR induction in this fraction may be from the unidentified components present in this isolate.

A few lipids, such as β-carotene and xanthophylls, have been identified as QR inducing agents (*2*). Most lipids do not have characteristics common to QR inducers, such as sulfhydryl reactivity and the presence of Michael reaction acceptor groups. However, near 120 μM, fatty acids isolated from soy could double QR at or near the IC_{50} value in vitro. At these levels, fatty acid and monoacylglycerols may act as surfactants, perturb cellular membrane, and evoke oxidative stress, resulting in induction of protective enzymes. Oxidized lipids are known to induce phase 2 enzymes (*28, 29*). Fatty acids have previously been shown to induce oxidative stress, membrane perturbation, DNA damage, and apoptosis in cells in vitro (*30, 31*).

FAEE was the most potent QR inducing agent identified in the 80% methanol soy isolate. It was more potent than genistein (CD value = 19 μM), and also FA (CD value = 1200 μM) and FAME (CD value = 15 μM). A free carboxylic acid interferes with the Michael reaction, which accounts for the reduced potency of phenolic acids compared to their esterified derivatives (*32*). Previous studies by Talalay's group have identified methyl-substituted hydroxycinnamates as moderately potent QR inducers (*2, 32*), but no previous reports could be found on how the nature of the ester impacts QR inducing potency. An ethyl substitution may enhance delivery through the cell membrane by increasing the lipophilic nature of the FA derivative. This correlates with other aspects of its antioxidant nature, as FAEE is more potent than FA in a lipophilic oxidative environment and ex vivo LDL oxidation models (*33, 34*).

FA has been reported as a neuroprotective agent, and consequently, FAEE has also been tested for similar in vivo efficacy (*35, 36*). In response to oxidative stress, neurons in

gerbils had increased levels of heme oxygenase-1 (HO-1) when injected with FAEE (*36, 37*). HO-1 is an antioxidant enzyme regulated by the antioxidant response element (ARE). This previous study demonstrated that FAEE can induce antioxidant enzymes in vivo. Because QR induction also requires the activation of the ARE, the prospects appear good that FAEE may also induce QR in vivo.

FA is one of the most abundant phenolics in foods. However, FAEE is found in trace amounts in a wide variety of plants and foods (*38, 39*). FAEE is hypothesized to be an intermediate in lignin synthesis and appears to comprise a small metabolic pool with regard to facilitating lignin cross-linking (*38*). The ubiquity of ferulic acid and the potential to elevate intrinsic levels of FAEE in plant foods comprise an area that warrants attention for the purpose of enhancing health-promoting properties of foods.

Although 10 peaks were recovered from TLC band 3, only 5 contained enough mass for analysis of QR-inducing activity. None of the peaks that were tested were as potent as the parent isolate. It is possible that the potent QR inducers were the trace or unidentified elements in this fraction or that they act synergistically. Benzofuran-3-carboxylic acid ethyl ester was identified as peak 2 of band 3. A standard compound could not be obtained for authentication purposes. As it had a CD value of 40 μg/mL, it is unlikely that peak 2 or any other unidentified components contributed to the potency associated with material comprising this band. The benzofuran structure lacks electrophilic sites, which are required for activation of the ARE.

From TLC band 4, peaks 4 and 5 were of similar potency as the parent fraction 2. Peak 4 had the greatest IUs recovered from TLC band 4, but could not be identified further, as ESI and EI mass spectra were inconsistent. NMR experiments also indicated a low purity, and further resolution of peak 4 would need to be obtained before identification can be accomplished. Peak 4 contributed 45% of the recovered IUs from TLC band 4 (**Table 4**) and about 15% IUs collectively recovered from TLC bands 2−4. 4-Hydroxybenzaldehyde was identified as peak 1 resolved from TLC band 4. A known volatile in wines, 4-hydroxybenzaldehyde may not be a common phenolic in soy, as one study detected the presence of this compound, whereas another did not (*40, 41*). Peak 1 had moderate inducing potency, but the purified 4-hydroxybenzaldehyde had weaker inducing ability, as it doubled QR specific activity at 21 μg/mL. The unknown impurity detected in peak 1 may be responsible for the enhanced potency of this isolate over pure 4-hydroxybenzaldehyde.

Benzofuran-3-carbaldehyde, isolated from TLC band 4 of fraction 2, is structurally related to the benzofuran-2-carboxylic acid ethyl ester identified in TLC band 3. Benzofurans have previously been identified in plant materials and are products of the dehydrodimerization of ferulic acid (*42, 43*). Benzofurans are known intermediates in lignin synthesis and have been reported in other plants (*44*). The Maillard browning reaction can also produce furans through condensation of amino acids and sugars, and benzofuran has been detected in the melanoidin fraction of thermally treated foods (*45*).

Peak 3 was identified as 4-hydroxybenzoic acid ethyl ester. It is an antifungal and antibacterial agent used in foods and cosmetics and has recently been reported as a tyrosinase inhibitor (*46*). As 4-hydroxybenzoic acid ethyl ester doubled the QR specific activity at 53 μg/mL, it may have a limited contribution to the overall QR inducing potency of soy isolates.

4-Ethoxycinnamic acid was the most potent of the TLC band 4 isolates, with a CD value of 6.3 μg/mL. This cinnamic acid derivative is unique as it has an ethoxy (ether) group and has not previously been isolated from soy. Also, the compound may have unique properties in that it was still a potent QR inducing agent even though it possesses a free carboxylic acid group. As discussed above, the improved lipophilicity of the compound may facilitate its QR inducing action in cells.

In the present study, QR-inducing agents were identified using the Hepa 1c1c7 cell line, but the metabolic fate of these agents and in vivo bioactivity remain to be determined. For example, human and rodent HepG2 and H4IIE cultured hepatocytes retain the ability to catalyze phase 2 enzyme reactions, such as the ability to glucuronidate, sulfate, and methylate hydroxyl groups of the flavonoid quercetin (*3*). However, in vitro studies are limited in the ability to model the dynamic process of absorption, delivery, and excretion of phytochemicals. For example, the isoflavone genistein was a potent QR inducer in the Hepa 1c1c7 cell line, but the most abundant conjugated forms of genistein in soy flour were weak QR inducers (*14*). Also, supplementation of purified genistein to rats did not cause induction of QR in liver tissues (*15, 16*). Therefore, the most promising phase 2 enzyme inducing agents, such as FAEE, should be evaluated for in vivo efficacy.

In conclusion, the high potency and low CI of FAEE suggest that it should be evaluated in vivo to determine dietary phase 2 enzyme inducing efficacy. FAEE and other minor constituents, many of which remain to be identified, may have greater potential to contribute to phase 2 enzyme induction of soy than isoflavones (*14*).

ABBREVIATIONS USED

QR, quinone reductase; MPLC, medium-pressure liquid chromatography; FA, ferulic acid, FAEE, ferulic acid ethyl ester; FAME, ferulic acid methyl ester; BSTFA, *N,O*-bis(trimethylsilyl) trifluoroacetamide; TCMS, trimethylchlorosilane; FID, flame ionization detector; AIC, Analytical Instrumentation Center; CID, chemically induced collision; HPBCD, hydroxypropyl β-cyclodextrin; EDTA, ethylenediaminetetraacetic acid; NADPH, nicotinamide-adenine dinucleotide phosphate, reduced; SDS, sodium dodecyl sulfate; MTT, 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2*H*-tetrazolium bromide; DMSO, dimethyl sulfoxide; NOESY, nuclear Overhauser effect spectrocopy; gDQCOSY, gradient double-quantum correlation spectroscopy; TOCSY, total correlation spectroscopy; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear multiple quantum coherence; APCI, atmospheric pressure chemical ionization; ESI, electrospray ionization; EI, electron ionization; IC_{50}, inhibitory concentration at 50%; CD, concentration required to double; CI, chemopreventive index; IUs, inducing units.

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Supporting Information Available: Flow diagram of soy flour extraction and purification process. This material is available free of charge via the Internet at http://pubs.acs.org.

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