Available online at www.sciencedirect.com
ScienceDirect
Phytochemistry 68 (2007) 1059–1067
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Isolation and identification of potential cancer chemopreventive agents from methanolic extracts of green onion (*Allium cepa*)

Hang Xiao [1], Kirk L. Parkin *

Department of Food Science, Babcock Hall, 1605 Linden Drive, University of Wisconsin-Madison, Madison, WI 53706

Received 6 October 2006; received in revised form 29 December 2006

Abstract

Phase II xenobiotic metabolizing enzymes confer amelioration of risk arising from potentially carcinogenic chemicals derived both endogenously, and exogenously, from food and the environment. In this study, efforts were made to isolate and identify potentially cancer preventive constituents from methanolic extracts of green onion (*Allium cepa*) directed by the quinone reductase (QR) induction bioassay using murine hepatoma (Hepa 1c1c7) cells. Crude methanolic extracts of green onion tissue were solvent-partitioned, and subsequently fractionated by flash chromatography, thin layer chromatography and high pressure preparative liquid chromatography to afford pure QR-inducing isolates. Multiple isolates were found active at inducing QR. One newly identified compound, 5-hydroxy-3-methyl-4-propylsulfanyl-5H-furan-2-one (**3**), and four known compounds: 5-(hydroxymethyl) furfural (**1**), acetovanillone (**2**), methyl 4-hydroxyl cinnamate (**4**) and ferulic acid methyl ester (**5**), were isolated and identified as active agents.

Keywords: *Allium cepa*; Alliaceae; Green onion; Phase II enzyme inducers; Quinone reductase; Glutathione; S-transferase; Cancer chemopreventive; Hepa 1c1c7 cells

1. Introduction

Epidemiological studies have provided strong evidence that a diet containing copious amounts of fruits and vegetables affords cancer risk reduction in humans (Block et al., 1992; Helzlsouer et al., 1994). A large number of studies on laboratory animals have also demonstrated that a wide range of nonnutritive dietary chemicals derived from fruit and vegetables inhibit chemical carcinogenesis caused by electrophiles and reactive oxygen species (ROS) arising from endogenous and exogenous sources. One mechanism responsible for the protective role of fruit and vegetable consumption is the induction of Phase II xenobiotic metabolizing enzymes, which include glutathione *S*-transferase (GST), quinone reductase (QR), and UDP-glucuronosyltransferase (UGT) (Talalay, 1989, 1992). Phase II enzyme induction has emerged as an important strategy for cancer chemoprevention (Talalay, 2000).

In an effort to facilitate the screening and identification of potential Phase II enzyme inducers, a rapid and direct bioassay of QR induction in Hepa 1c1c7 cells grown in microtiter plates was developed (Prochaska and Santamaria, 1988), and this bioassay has been recognized and used by numerous research laboratories worldwide (Kang and Pezzuto, 2004). As a representative Phase II enzyme, QR plays important roles in detoxifying harmful quinones (Dinkova-Kostova and Talalay, 2000) and acting as an antioxidant enzyme (Beyer, 1994; Beyer et al., 1997; Ross et al., 2000; Siegel et al., 1997). QR deficiency in humans and laboratory animals is associated with increased risk of various malignancies (Krajinovic et al., 2002; Long et al., 2001; Radjendirane et al., 1998; Rothman et al., 1997; Smith et al., 2001).

Allium consumption has been inversely related to stomach and colorectal cancer through epidemiological studies, and this suggests a chemoprotective role of high intakes of

* Corresponding author. Tel.: +608 263 2011; fax: +608 262 6872.
E-mail address: klparkin@wisc.edu (K.L. Parkin).
[1] Department of Chemical Biology & Center for Cancer Prevention Research, Earnest Mario School of Pharmacy, Rutgers, The State University of New Jersey, United States.

0031-9422/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2007.01.021

Allium vegetables such as onions and garlic (Ernst, 1997; Fleischauer and Arab, 2001; Le Bon and Siess, 2000). Phytochemicals often studied from *Allium* vegetables, mainly organosulfur compounds, have been shown to induce Phase II enzymes in multiple organs and tissues in animal models (Guyonnet et al., 1999; Haber et al., 1995; Manson et al., 1997; Reddy et al., 1993; Siess et al., 1997; Sparnins et al., 1988; Sumiyoshi and Wargovich, 1990), and this effect may constitute one means by which diets rich in *Allium* vegetables confer protection against cancer. Onions and related *Allium* crops are among the most widely consumed vegetables on a global basis. In a previous study (Xiao and Parkin, 2006), isolates from non-polar ethyl acetate extract (Fig. 1) of green onion were shown to possess potent QR-inducing activities in vitro, and among the five compounds isolated (ferulic acid, *p*-hydroxyphenethyl *trans*-ferulate, *N-trans*-feruloyl 3-*O*-methyldopamine, 5,6-dimethyl-2-pyridinecarboxylic acid, and 1-(6-hydroxy-[3]pyridyl-propan-1-one), none was an organosulfur compound. Interestingly, many studies on health benefits of Alliums dogmatically infer that organosulfur compounds are the responsible agents.

This study represents a continued effort to isolate and identify potential chemopreventive agents from green onion tissue, using the QR-induction bioassay to direct the isolation of Phase II enzyme inducers.

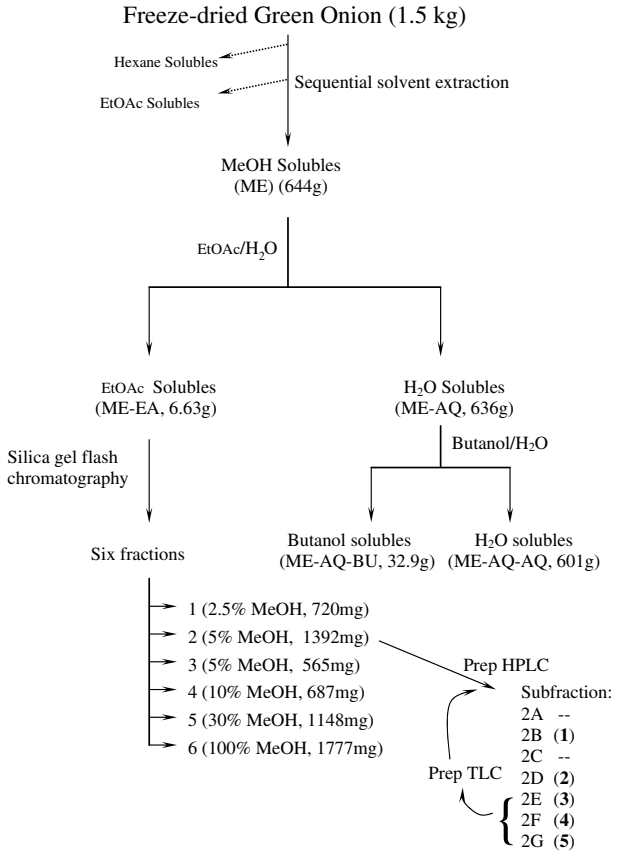

Fig. 1. Extraction and isolation scheme for phase II enzyme inducing components.

2. Results and discussion

2.1. QR-inducing activity of crude solvent extracts

Three crude solvent extracts, ME-EA, ME-AQ-BU and ME-AQ-AQ, were afforded (Fig. 1) by water–butanol partitioning of the MeOH extract of green onion (*Allium cepa*) tissue powder that had been previously extracted by hexane and ethyl acetate (Xiao and Parkin, 2006). These three fractions were then subjected to a QR induction assay using Hepa 1c1c7 cells. The concentration required for doubling the specific activity of QR (CD value), relative to non-treated control cells, was used as an indicator of inducer potency, and the minimum concentration required to cause $\geqslant 50\%$ reduction in cell protein was defined as the IC_{50} value. The relative potency of QR inducing activity of isolates from the crude MeOH extract was ME-EA $\gg$ ME-AQ-BU > ME-AQ-AQ ($p < 0.05$; Table 1). Solvent partitioning effectively concentrated QR inducing activity of the crude ME extract into the ME-EA fraction that could double QR activity at 12.6 μg/ml with a 4-fold margin between CD and IC_{50} (48.2 μg/ml) values.

2.2. QR inducing activity of fractions derived from the ME-EA isolate

Six fractions were yielded from silica gel flash chromatography (Fig. 1), and they were subjected to the QR bioassay. Only fractions eluting at 2.5% and 5% MeOH had potent inducing power (CD < 15 μg/ml) towards QR (Table 1). Total recovery of mass and QR inducing activity

Table 1
QR inducing activities of crude extracts and fraction obtained from ME-EA

Test sample	CD ± SD (μg/ml)	IC_{50} ± SD (μg/ml)	IC_{50}:CD
Crude solvent extracts			
ME-EA	12.6 ± 1.1	48.2 ± 2.7	3.82
ME-AQ-BU	201 ± 18	>1000	>5
ME-AQ-AQ	>1000	>1000	NA
Fractions of ME-EA			
1 (2.5% MeOH)	6.70 ± 1.05	11.9 ± 1.5	1.78
2 (5% MeOH, 1st portion)	14.1 ± 1.1	40.0 ± 8.0	2.84
3 (5% MeOH, 2nd portion)	22.4 ± 0.7	74.7 ± 21.3	3.33
4 (10% MeOH)	29.2 ± 5.0	127 ± 6	4.35
5 (30% MeOH)	20.9 ± 2.4	84.1 ± 21.2	4.02
6 (100% MeOH)	56.7 ± 5.4	194 ± 17	3.42

CD and IC_{50} are reported as means ± SD of at least three replicates. All values were significantly different ($p < 0.01$) from the controls by a two-tailed Student's *t*-test. NA is not applicable as the isolate did not double QR specific activity. Italicised are isolates on which further purifications were performed.

from the parent ME-EA isolate among the six fractions was 95% and 65%, respectively. Although fraction ME-EA-1 had a CD value of 6.7 μg/ml, an analytical HPLC profile revealed a complexity (Fig. 2) and anticipated difficulty in efforts at further isolation to render this isolate a lower priority than others. Fraction ME-EA-2 doubled QR activity in Hepa 1c1c7 at about 14 μg/ml without significant cell growth inhibition (IC_{50}: 40 μg/ml), and its abundance was ~2 times greater than that of fraction ME-EA-1. Moreover, the HPLC chromatogram of fraction ME-EA-2 revealed a rather simple profile (Fig. 3), making the prospects favorable for further purification. Reversed phase flash chromatography was then performed on fraction ME-EA-2 and the chromatogram is shown in

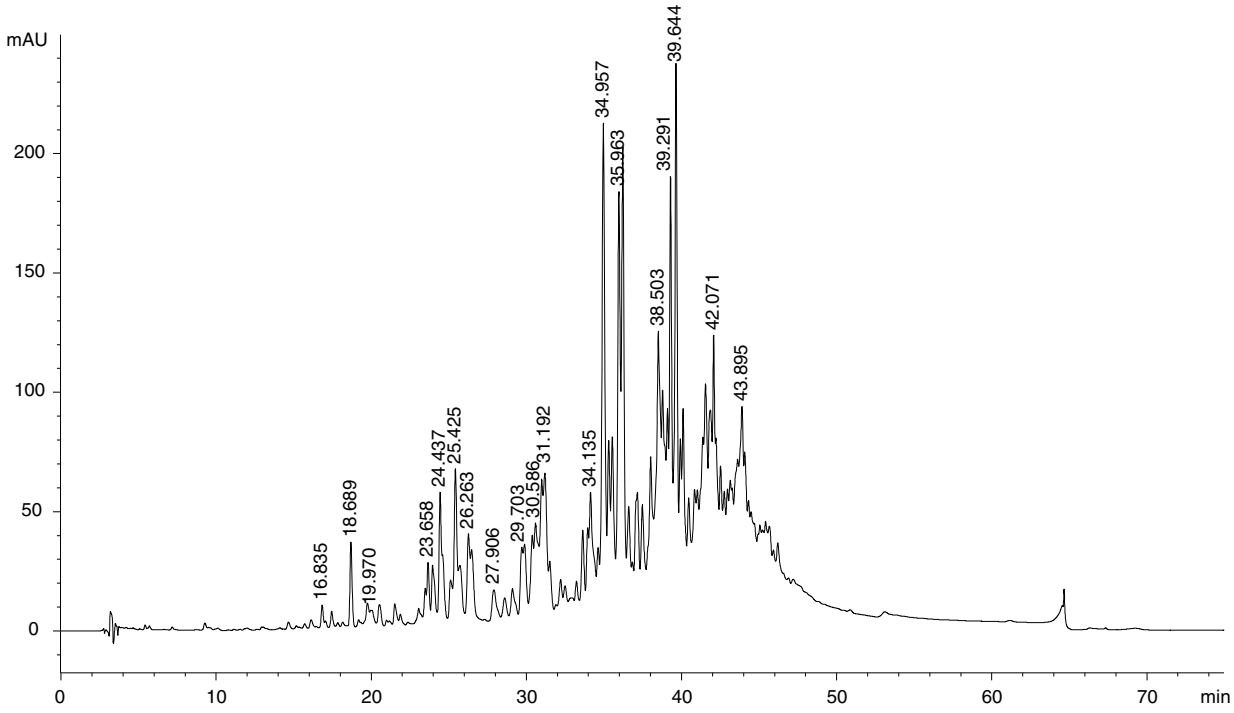

Fig. 2. Reversed phase HPLC chromatogram of fraction 1 derived from the ME-EA isolate (detection at 285 nm).

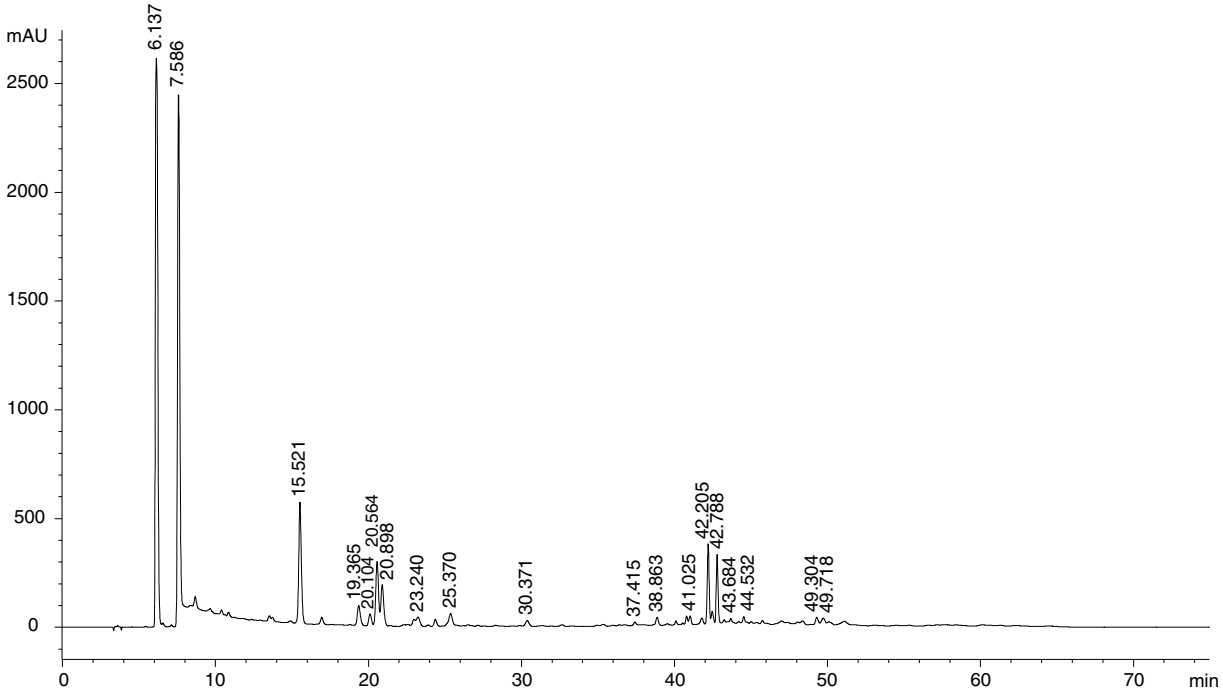

Fig. 3. Reversed phase HPLC chromatogram of fraction 2 derived from the ME-EA isolate (detection at 285 nm).

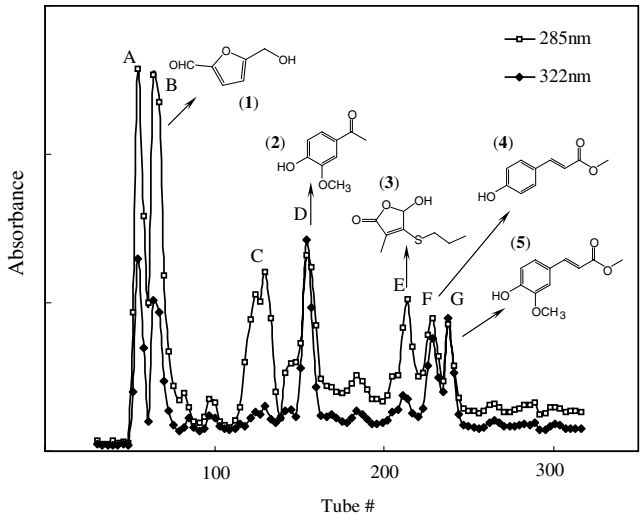

Fig. 4. Flash chromatogram of fraction 2 derived from the ME-EA isolate. Reversed phase flash chromatography on C18 silica gel (2.5 × 60 cm), linear gradient from 2% to 30% of CH_3CN in 1% AcOH within 288 min, flow rate 8 ml/min, fractions collected in 8 ml/tube.

Fig. 4. A total of seven subfractions were collected and their QR-inducing activities are shown in Table 2. All subfractions except ME-EA-2A and ME-EA-2B exhibited potent QR inducing potency with CD values of <10 μg/ml. All subfractions except ME-EA-2A exhibited a ratio of IC_{50}:CD values of 4–14, indicating a considerable margin between QR inducing and cell growth inhibitory effects. Subsequent preparative HPLC of these subfractions (ME-EA-2B, and ME-EA-2D through ME-EA-2G afforded five pure compounds **1**–**5** (Fig. 4). Subfractions ME-EA-2A and -2C were not explored further because of complexity and lack of a dominant component as revealed by analytical HPLC (chromatograms not shown).

2.3. Identification of isolated pure compounds

Compounds **1**, **2**, **4**, and **5** were identified as 5-(hydroxymethyl) furfural, 1-(4-hydroxy-3-methyl-phenyl)-ethanone (acetovanillone), methyl 4-hydroxyl cinnamate, and ferulic acid methyl ester, based on their spectroscopic properties. Their NMR spectra were consistent with those of authentic compounds, and significant HMBC correlations are shown in Fig. 5.

Table 2
QR inducing activity of subfractions derived from fraction 2 obtained from the ME-EA isolate

Subfractions	CD ± SD (μg/ml)	IC_{50} ± SD (μg/ml)	IC_{50}:CD
2A	19.3 ± 1.8	19.9 ± 5.2	1.03
2B	31.5 ± 4.0	195 ± 16	6.19
2C	6.26 ± 1.31	90.0 ± 1.4	14.4
2D	9.12 ± 0.61	57.0 ± 1.8	6.25
2E	2.07 ± 0.33	21.5 ± 0.1	10.4
2F	3.39 ± 1.40	14.2 ± 1.4	4.19
2G	5.14 ± 4.98	26.7 ± 2.1	5.19

CD and IC_{50} are reported as means ± SD of at least three replicates. All values were significantly different ($p < 0.01$) from the controls by a two-tailed Student's *t*-test.

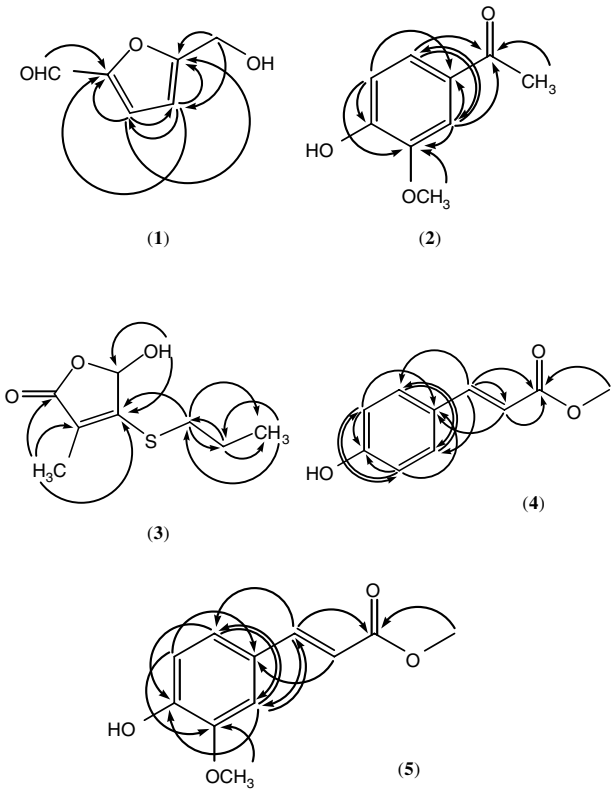

Fig. 5. Significant proton-carbon long-range correlations observed from HMBC spectra.

Compound **3** was a compound not previously reported and identified as 5-hydroxy-3-methyl-4-propylsulfanyl-5*H*-furan-2-one. EI and ESI mass spectra showed $[M]^+$ and $[M+Na+MeOH]^+$ at *m/z* 188 and 243, respectively. Subsequent high-resolution exact mass measurements on those two ions (188.0504 for $[M]^+$, and 243.0667 for $[M+Na+MeOH]^+$) suggested the molecular formula to be $C_8H_{12}O_3S$, which was supported by ^{13}C spectrum (eight signals). A $-CH_2CH_2CH_3$ structural element was deduced from three methyl protons (δ_H 1.06), two sets of methylene protons (2H each at δ_H 1.73 and 3.08) and their HMBC correlations with corresponding alkyl carbons at δ_C 13.2, 23.4 and 32.4. From HMQC and HMBC spectra, the second set of methyl protons (δ_H 1.86) had correlations with three quaternary carbons C-2 (δ_C 170.0), C-3 (δ_C 123.2), and C-4 (δ_C 156.9), which positioned this methyl group on C-3. Based on the molecular formula and chemical shift of methylene carbon (δ_C 32.4) that had correlation with C-4, the sulfur atom was suggested to be between a methylene carbon (δ_C 32.4) and C-4. The correlations between the hydroxyl proton (δ_H 3.3) and C-4 and C-5 were consistent with the overall substitution assignment on the ring structure.

2.4. Phase II enzyme inducing activities of pure isolates

Compounds **1**–**5** were tested for inducing activity on QR and GST (Table 3). Collectively, these pure isolates repre-

Table 3
Phase II induction by pure isolates

Compound	Dose range (μM)	IC_{50} (μM)	Quinone reductase			Glutathione *S*-transferase	
			I_{max} (μM)	CI_{max} (μM)/(μg/ml)	CD (μM)/(μg/ml)	I_{max} (μM)	CI_{max} (μM)/(μg/ml)
1	15–7665	997 ± 48	1.60 ± 0.05	958/121	NA	1.55 ± 0.15	958/121
2	3.5–1775	1060 ± 7	1.55 ± 0.09	888/148	NA	1.74 ± 0.08	888/148
3	10–5318	890 ± 159	5.55 ± 0.37	665/125	83.0 ± 9.2 /15.6 ± 1.7	2.10 ± 0.26	665/125
4	2–1038	115 ± 7	3.68 ± 0.50	65/12	20.4 ± 2.0/3.6 ± 0.4	1.69 ± 0.24	109/19.4
5	2.6–1346	368 ± 34	3.91 ± 0.22	337/70	15.4 ± 0.5/3.19 ± 0.11	1.62 ± 0.33	337/70

IC_{50}, minimum concentration required to cause ⩾50% reduction of cell survival; I_{max}, maximum induction ratio; CI_{max}, concentration for maximum induction; CD, concentration required for doubling enzyme activity; NA, not applicable (isolate did not double QR specific activity). Results are reported as means ± SD of at least three replicates. All values were significantly different ($p < 0.01$) from the controls by a two-tailed Student's *t*-test.

sented a recovery of only about 2.5% of the mass and ⩾2.6% QR inducing capacity of the parent fraction ME-EA-2. Unavoidable losses during purification make it difficult to assess to what extent these compounds account for the QR inducing activity of the crude extracts of green onion. 5-Hydroxymethyl furfural (**1**) and acetovanillone (**2**) weakly induced both QR and GST activity and failed to double their activities at concentrations up to their IC_{50} values (~1000 μg/ml). 5-Hydroxy-3-methyl-4-propylsulfanyl-5H-furan-2-one (**3**) induced QR activity up to 5.55-fold compared to the control, and it had a CD value of 83 μM (15.6 μg/ml). Moreover, compound **3** also doubled GST activity at the upper end of the dose range tested. Methyl 4-hydroxyl cinnamate (**4**) and ferulic acid methyl ester (**5**) induced QR up to 3.68- and 3.9-fold compared to the control, and they doubled QR at 20.4 μM (3.6 μg/ml) and 15.4 μM (3.19 μg/ml), respectively. These two compounds modestly induced GST activity by about 1.6-fold. A previous report showed methyl 4-hydroxyl cinnamate (**4**) and ferulic acid methyl ester (**5**) to have the same CD value of 83 μM (Dinkova-Kostova et al. 1998), a value 4.1- and 5.4-fold greater than the respective CD values found in this study. One possible explanation for this discrepancy is that hydroxypropyl-β-cyclodextrin (HPBC) was used in this study as a delivery system for lipophilic samples in cell culture medium to increased dispersibility of **4** and **5** and this may be responsible enhanced inducing potency. HPBC has been tested as an alternative to organic solvent for delivering poorly water-soluble substances, triiodothyronine and thyroxine, to rat hepatocytes in a study for their interference of GST expression (Vanhaecke et al., 2001). This study indicated that HPBC is an effective vehicle to carry hydrophobic molecules into aqueous media.

Compounds **1** and **2** did not double QR activity at concentrations up to their IC_{50} values (121 and 148 μg/ml, respectively), while the parent subfractions, ME-EA-2B and ME-EA-2D, had CD values of 31.5 and 9.12 μg/ml, respectively. This suggested that compounds **1** and **2** might not be the most active agents in the parent subfractions, or that synergy among active agents was lost during fractionation. Although compound **3** doubled QR activity, its CD value was more than 7-fold higher than that of its parent subfraction, ME-EA-2E. Moreover, the IC_{50} of the subfraction ME-EA-2E was about 8-fold lower than that of compound **3**, indicating the presence of other active components in this subfraction besides compound **3**. Compounds **4** and **5** had CD values (3.6 and 3.19 μg/ml) of a similar magnitude as that of their parent subfractions (3.39 and 5.14 μg/ml), ME-EA-2F and ME-EA-2G, which indicated that compounds **4** and **5** may be largely responsible for the inducing activities of these parent subfractions.

Compounds **4** and **5** are *p*-coumaric (hydroxycinnamic) acid derivatives, which have α,β-unsaturated carbonyl functional groups that constitute "Michael acceptors". Michael acceptor types of structures have been found in many Phase II enzyme inducers and their inducing potencies closely correlate with their Michael reactivities (Talalay, 1989). Michael reactivity depends on the nature of electron-withdrawing group conjugated with olefin structure. In general the reactivity decreases as electron-withdrawing group = COAr > CHO > $COCH_3$ > CO_2CH_3 > CN > CONH2 > > COOH. Compounds **4** and **5** have a $-CO_2CH_3$ type of electron-withdrawing group. The only structural difference between compounds **4** and **5** is the *m*-methoxy group on the benzene ring that apparently did not affect QR-inducing potency much, but made compound **5** more than 3-times less growth inhibitory than compound **4** (IC_{50}: 368 μM versus 115 μM). Substituted phenolic groups tend to be less potent inducers that the corresponding free phenolic derivatives (De Long et al., 1986).

In our previous study on nonpolar extracts of green onion, *p*-hydroxyphenethyl *trans*-ferulate and *N*-*trans*-feruloyl 3-*O*-methyldopamine were indentified as QR inducers with respective CD values of 6.6 and 61 μM (Xiao and Parkin, 2006). Similar to compound **5**, these compounds have a ferulic acid moiety in their structures. However, ferulic acid and *N*-*trans*-feruloyl 3-*O*-methyldopamine were less potent inducers than compound **5**, apparently because their electron-withdrawing groups produce weak Michael reactivities.

3. Conclusions

Many lines of evidence have supported the premise that induction of Phase II enzymes affords protection against neoplastic effects of chemical carcinogens. An in vitro bioassay in Hepa 1c1c7 cells based on QR induction has been effectively used as a tool for screening potential cancer

chemopreventive agents from plant materials, leading to the discovery of sulforaphane as an inducer from broccoli (Prochaska et al., 1992). Using this bioassay, extracts of green onion were identified as the most potent QR inducing isolate among 28 different fruit and vegetable extracts (Prochaska et al., 1992). However, the identity of the inducing agents derived from green onion extracts has not followed until this and a related report (Xiao and Parkin, 2006). Our results indicated that solvent partitioning effectively concentrated QR inducing activity of methanolic extracts of green onion tissue into a single fraction (ME-EA) where multiple subfractions with potent inducing power were obtained by further chromatographic purification. Five pure compounds were isolated and identified and in vitro QR inducers, including one new compound, 5-hydroxy-3-methyl-4-propylsulfanyl-5H-furan-2-one (**3**). The possibility exists that other inducers with stronger or synergistic inducing power are present in active subfractions, and this warrants further research to isolate and identify these agents for their potential for Phase II enzyme induction in vitro and in vivo.

4. Experimental

4.1. Materials and general experimental procedures

Acetovanillone and 5-(hydroxymethyl) furfural were purchased from Aldrich Chemical Co. (Milwaukee, WI). Methyl 4-hydroxycinnamate was obtained from Frinton Laboratories (Vineland, NJ). Ferulic acid methyl ester was obtained from Senn Chemicals (Dielsdorf, Switzerland). 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 purchased from Gibco (Grand Island, NY). All other chemicals were from Sigma Chemical Co. (St. Louis, MO). Solvents were procured from Fisher Scientific (Fair Lawn, NJ).

Freeze-dried green onion was purchased as a single lot from Van Drunen Farm (Momence, IL). This material was identified by the supplier to be comprised of *Allium cepa* (no certification was provided).

Thin-layer chromatography (TLC) was conducted on Whatman silica gel (60 Å) UV_{254} plates (layer thickness: 250 μm for analytical and 1000 μm for preparative), with compounds visualized under $UV_{254\,nm}$ illumination and/or by staining with iodine vapor (Aldrich, Milwaukee, WI). Silica gel (60 Å, 230–400 mesh ASTM, Fisher Scientific) and C18 reversed phase silica gel (60 Å, 230–400 mesh ASTM, EMD Chemical Inc., Gibbstown, NJ) were used for flash column chromatography (CC) conducted with Chromaflex columns (Kontes, Vineland, NJ) equipped with a pump, a gradient former (Model 160, ISCO, Lincoln, NE), and a fraction collector (Foxy Jr., ISCO). HPLC analysis was conducted using a Discovery C18 column (5 μm, 25 cm × 4.6 mm, 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 system (Model 2300 pumps, V^4 detector, and with peak integration by an integrator (Model SP4270, Spectra-Physics, San Jose, CA) using a Discovery C18 column (5 μm, 25 cm × 21.2 mm, Supelco).

4.2. 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 spectroscopy experiments were performed using standard pulse sequences. The instruments are housed, operated and maintained in the School of Pharmacy at University of Wisconsin-Madison.

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. The nominal mass spectra were obtained using a direct sample probe, and the magnet was scanned from *m/z* 1000 to *m/z* 35 (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 experiments were performed on either a Waters LCT Electrospray-TOF instrument, or on 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 polyethylene glycol peaks as known masses. All MS analyses were carried out in the mass spectrometry facility of the Department of Chemistry at the University of Michigan (Ann Arbor).

4.3. 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 (Prochaska et al., 1992; Xiao and Parkin, 2006). Fetal bovine serum was treated with activated charcoal to remove any traces of endogenous QR inducers prior to use. Cells were placed in 96-well plates at an inoculum of 5000 cells per well, grown for 24 h and then the test isolates were added (in 150 μl MEM). The cells were induced for an additional 48 h at 37 °C in 5% CO_2 in air. Test samples with low solubility were dispersed in MEM containing 0.1% of HPBC or DMSO (final concentration <0.5%) prior to adding to the cells. Corresponding controls without test isolates were

prepared in all cases and HPBC and DMSO had no effect on QR induction at the levels used.

After decanting culture medium, cells were lysed by adding 50 μl of 0.08% (w/v) aqueous digitonin. QR activity was measured after adding 200 μl of a standard assay cocktail to each well (Prochaska et al., 1992; Xiao and Parkin, 2006). The absorbance of the reduced tetrazolium dye was measured over a 10-min period using an optical microtiter plate scanner (Spectra Max plus, Molecular Devices, Sunnyvale, CA) set at 490 nm. A duplicate plate was prepared for cell protein (viability) measurement and to facilitate enzyme specific activity calculation as described previously (Xiao and Parkin, 2006). The concentration required for doubling the specific activity of QR (CD value) relative to non-treated control cells was used as an indicator of inducer potency.

4.4. Glutathione S-transferase (GST) assay

Hepa 1c1c7 cells were cultured, treated with test samples, and then lysed in the same way as just described. GST activity was measured by an established method adapted to 96-well plates (Habig et al., 1974; Xiao and Parkin, 2006). Stock solution (150 μl) containing 1.33 mM of 1-chloro-2,4-dinitrobezene (CDNB), 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 analysis.

4.5. Cell protein (viability) assessment

Samples from a replicate 96 well plate were decanted after the 48-h induction period and assayed for protein by incubation with 100 μL of crystal violet (0.2% in 2% ethanol) for 10 min followed by washing off free dye (lukewarm tap water). The bound dye was solublized by adding 150 μl of 0.5% (w/v) SDS solution (prepared in 50% aqueous ethanol) and after incubation for 1 h in a shaker oven set at 37 °C, absorbance of each well was measured at 610 nm. The degree of staining with crystal violet was used as a measure of cell protein. A cell growth inhibitory concentration (IC_{50}) was determined as the minimum level required to decrease cell protein by $\geqslant 50\%$ relative to untreated control cells.

4.6. Statistical analysis

Results are reported as mean values ± standard deviation (SD) from at least three replicates. The differences among treatment means were evaluated by a one-way ANOVA and a two-tailed Student's *t*-test.

4.7. Extraction and isolation

4.7.1. Crude extracts

Freeze-dried green onion (1.50 kg) was sequentially extracted with hexane and ethyl acetate as described previously (Xiao and Parkin, 2006), and then the residual material was extracted for 8 h with anhydrous MeOH using an over-size Sohxlet apparatus (extraction chamber: 10 × 40 cm). After drying in vacuo at 50 °C, the MeOH extract (ME, 644 g) was suspended in H_2O (1 l) and then washed with EtOAc (5 × 1 l) to afford an EtOAc-partitioned fraction (ME-EA 6.63 g) and a H_2O-partitioned fraction (ME-AQ, 636 g). The ME-AQ fraction was further and thrice partitioned against an equal volume of *n*-BuOH to generate two additional fractions: ME-AQ-BU (32.9 g) and ME-AQ-AQ (601 g). Solvents were removed by evaporation in vacuo at 50 °C or by lyophilization. Subsequent QR bioassay revealed that only the ME-EA fraction was sufficiently active to warrant further fractionation.

4.7.2. Resolution of the ME-EA isolate

The ME-EA isolate was fractionated by flash CC (4.8 × 30 cm column) with silica gel as stationary phase using a five-step gradient of 2.5%, 5%, 10%, 30%, and 100% MeOH in CH_2Cl_2 at flow rate of 15 ml/min, with each step comprising a run time of 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 spectral data collected between 210 nm to 340 nm. Six pooled fractions were obtained (Fig. 1) and fraction ME-EA-2 was subjected to further isolation based on its QR-inducing potency, relative abundance, and degree of resolution as judged from an analytical HPLC profile (Fig. 3).

Fraction ME-EA-2 (the first fraction eluted by 5% MeOH in methylene chloride) obtained from the ME-EA isolate (1.39 g) was applied to a reverse phase flash chromatography column (C18 silica gel, 2.5 × 60 cm column), and resolved by a linear gradient from 2% to 30% of acetonitrile in 1% acetic acid within 288 min (flow rate 8 ml/min) to afford 7 subfractions: ME-EA-2A to ME-EA-2G (Fig. 4). Further purification by reverse phase preparative HPLC (flow rate 7 ml/min) of isolate ME-EA-2B (isocratic 2% acetonitrile in 1% acetic acid), ME-EA-2D (isocratic 15% acetonitrile in 1% acetic acid), and ME-EA-2E, ME-EA-2F and ME-EA-2G (isocratic 25% acetonitrile in 1% acetic acid) afforded compounds (**1**) (15 mg), (**2**) (5 mg), (**3**) (9 mg), (**4**) (2 mg), and (**5**) (5 mg), respectively. Subfractions ME-EA-2E, ME-EA-2F and ME-EA-2G required additional cleanup by preparative TLC (30% ethyl acetate in toluene as the developing solvent) prior to re-chromatography by preparative HPLC to afford purification (Fig. 1).

4.8. Spectroscopic data of isolated compounds

4.8.1. 5-(Hydroxymethyl) furfural (**1**)

HREI *m/z*: 126.0323 (calcd. for $C_6H_6O_3$, 126.0317); EI (70 eV) *m/z* (rel. int.): 126 M^+ (74), 109 $[M–OH]^+$ (14), 97 $[M–CHO]^+$ (100), 69 (33), 53 (19), 41 (78), 39 (43); ^1H and ^{13}C NMR spectra were consistent with authentic compound obtained commercially.

4.8.2. 1-(4-Hydroxy-3-methyl-phenyl)-ethanone (acetovanillone) (**2**)

HREI *m/z*: 166.0631 (calcd. for $C_9H_{10}O_3$, 166.0629). EI (70 eV) *m/z* (rel. int.): 166 M^+ (58), 151 $[M–Me]^+$ (100), 123 $[M–COMe]^+$ (24), 108 $[M–COMe–Me]^+$ (4), 43 $[COMe]^+$(7). ^1H and ^{13}C NMR spectra were consistent with authentic compound obtained commercially.

4.8.3. 5-Hydroxy-3-methyl-4-propylsulfanyl-5H-furan-2-one (**3**)

HREI *m/z*: 188.0504 (calcd. for $C_8H_{12}O_3S$, 188.0507); EI (70 eV) *m/z* (rel. int.): 188 M^+ (69), 170 $[M–H_2O]^+$ (15), 128 [M–propyl–OH]$^+$ (16), 114 [M+H–propyl–OH–CH_3]$^+$ (100), 99 (73), 72 (51), 41 (43); HRESI (NaCl and MeOH added) *m/z*: 243.0668 (calcd. for $C_9H_{16}O_4SNa$ [M+Na+MeOH]$^+$, 243.0667); ESI (NaCl and MeOH added) *m/z*: 399 $[2M+Na]^+$ (59), 243 [M+Na+MeOH]$^+$ (100), 211 $[M+Na]^+$ (16), 189 $[M+H]^+$ (3); ^1H NMR ($CDCl_3$, 500 MHz): δ 6.04 (1H, *d*, $J = 9.5$ Hz, H-5), 3.30 (1H, *d*, $J = 9.5$ Hz, OH), 3.08 (2H, *m*, $CH_2CH_2CH_3$), 1.86 (3H, *s*, CH_3), 1.73 (2H, *m*, $CH_2CH_2CH_3$), 1.06 (3H, *t*, $J = 7.5$ Hz, $CH_2CH_2CH_3$); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 170.0 (C-2), 156.9 (C-4), 123.2 (C-3), 96.0 (C-5), 32.4 (SCH_2), 23 4 (CH_2CH_3), 13.2 (CH_2CH_3), 9.2 (CH_3).

4.8.3. Methyl 4-hydroxyl cinnamate (**4**)

HREI *m/z*: 178.0630 (calcd. for $C_{10}H_{10}O_3$, 178.0630); EI (70 eV) *m/z* (rel. int.): 178 M^+ (73), 147 (100), 119 (39), 91 (29), 65 (19); ^1H and ^{13}C NMR spectra were consistent with authentic compound obtained commercially.

4.8.4. Ferulic acid methyl ester (**5**)

HREI *m/z*: 208.0727 (calcd. for $C_{11}H_{12}O_4$, 208.0735); EI (70 eV) *m/z* (rel. int.): 208 M^+ (100), 193 $[M–Me]^+$(6), 177 $[M–OMe]^+$ (60), 147 [M–2(OMe)+H]$^+$ (28), 145 [M–2(OMe)–H]$^+$ (28), 133 (11), 117 (12), 89 (13), 51 (9); ^1H and ^{13}C NMR spectra were consistent with authentic compound obtained commercially.

Acknowledgments

This work was supported by the College of Agricultural and Life Sciences of the University of Wisconsin-Madison, in part through Grants from the Experiment Station (Hatch, WIS04787), and USDA-CGR-NRI (2005-35503-16328). We thank Dr. Thomas Stringfellow of the Analytical Instrumentation Center at the School of Pharmacy at the University of Wisconsin-Madison for his technical support.

References

Beyer, R.E., 1994. The relative essentiality of the antioxidative function of coenzyme Q-the interactive role of DT diaphorase. Mol. Aspects Med. 15, s117–s129.

Beyer, R.E., Segura-Aguilar, J., Bernardo, S.D., Cavazzoni, M., Fato, R., Fiorentini, D., Galli, M.C., Setti, M., Landi, L., Lenza, G., 1997. The two-electron quinone reductase generates and maintains the antioxidant (reduced) form of coenzyme Q in membranes. Mol. Aspects Med. 18 (Suppl.), s15–s23.

Block, G., Patterson, B., Subar, A., 1992. Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. Nutr. Cancer. 18 (1), 1–29.

De Long, M.J., Prochaska, H.J., Talalay, P., 1986. Induction of NAD(P)H:quinone reductase in murine hepatoma cells by phenolic antioxidants, azo dyes, and other chemoprotectors: a model system for the study on anticarcinogens. Proc. Natl. Acad. Sci. USA 83, 787–791.

Dinkova-Kostova, A.T., Talalay, P., 2000. Persuasive evidence that quinone reductase type 1 (DT diaphorase) protects cells against the toxicity of electrophiles and reactive forms of oxygen. Free Radical Biol. Med. 29, 231–240.

Dinkova-Kostova, A., Abeygunawardana, C., Talalay, P., 1998. Chemoprotective properties of phenylpropenoids, bis(benzylidene)cycloalkanones, and related Michael reaction acceptors: correlation of potencies as phase 2 enzyme inducers and radical scavengers. J. Med. Chem. 41 (26), 5287–5296.

Ernst, E., 1997. Can Allium vegetables prevent cancer? Phytomedicine 4 (1), 79–83.

Fleischauer, A., Arab, L., 2001. Garlic and cancer: a critical review of the epidemiologic literature. J. Nutr. 131 (3s), 1032s–1040s.

Guyonnet, D., Siess, M.H., Le Bon, A.M., Suschetet, M., 1999. Modulation of phase II enzymes by organosulfur compounds from allium vegetables in rat tissues. Toxicol. Appl. Pharmacol. 154 (1), 50–58.

Haber, D., Siess, M.H., Canivenc-Lavier, C., Le Bon, A.M., Suschetet, M., 1995. Differential effects of dietary diallyl sulfide and diallyl disulfide on rat intestinal and hepatic drug-metabolizing enzymes. J. Toxicol. Environ. Health 44 (4), 423–434.

Habig, W., Pabst, M., Jakoby, W., 1974. Glutathione *S*-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249 (22), 7130–7139.

Helzlsouer, K., Block, G., Blumberg, J., Diplock, A., Levine, M., Marnett, L., Schulplein, R., Spence, J., Simic, M., 1994. Summary of the round table discussion on strategies for cancer prevention: diet, food, additives, supplements, and drugs. Cancer Res. 54 (7 Suppl.), 2044s–2051s.

Kang, Y., Pezzuto, J., 2004. Induction of quinone reductase as a primary screen for natural product anticarcinogens. Methods Enzymol. 382, 380–414.

Krajinovic, M., Sinnett, H., Richer, C., Labuda, D., Sinnett, D., 2002. Role of NQO1. MPO and CYP2E1 genetic polymorphisms in the susceptibility to childhood acute lymphoblastic leukemia. Int. J. Cancer 97, 230–236.

LeBon, A., Siess, M., 2000. Organosulfur compounds from Allium and the chemoprevention of cancer. Drug Metabol. Drug Interact. 17 (1–4), 51–79.

Long, D.J., Waikel, R., Wang, X., Roop, D., Jaiswal, A., 2001. NAD(P)H:quinone oxidoreductase 1 deficiency and increased susceptibility to 7,12-dimethylbenz[*a*]-anthracene-induced carcinogenesis in mouse skin. J. Natl. Cancer Inst. 93 (15), 1166–1170.

Manson, M.M., Ball, H.W., Barrett, M.C., Clark, H.L., Judah, D.J., Williamson, G., Neal, G.E., 1997. Mechanism of action of dietary chemoprotective agents in rat liver: induction of phase I and II drug metabolizing enzymes and aflatoxin B1 metabolism. Carcinogenesis 18 (9), 1729–1738.

Prochaska, H.J., Santamaria, A.B., 1988. Direct measurement of NAD(P)H: quinone reductase from cells cultured in microtiter plate wells: A screening assay for anticarcinogenic enzyme inducers. Anal. Biochem. 169, 328–336.

Prochaska, H.J., Santamaria, A.B., Talalay, P., 1992. Rapid detection of inducers of enzymes that protect against carcinogens. Proc. Natl. Acad. Sci. USA 89, 2394–2398.

Radjendirane, V., Joseph, P., Lee, Y.H., Kimura, S., Klein-Szanto, A.J., Gonzalez, F.J., Jaiswal, A.K., 1998. Disruption of DT-diaphorase (NQO1) gene in mice leads to increased menadione toxicity. J. Biol. Chem. 273, 7382–7389.

Reddy, B.S., Rao, C.V., Rivenson, A., Kelloff, G., 1993. Chemoprevention of colon carcinogenesis by organosulfur compounds. Cancer Res. 53 (15), 3493–3498.

Ross, D., Kepa, J.K., Winski, S.L., Beall, H.D., Anwar, A., Siegel, D., 2000. NAD(P)H:quinone oxidoreductase 1 (NQO1): chemoprotection, bioactivation, gene regulation and genetic polymorphisms. Chemico-Biol. Interact. 129, 77–97.

Rothman, N., Smith, M.T., Hayes, R.B., Traver, R.D., Hoener, B., Campleman, S., Li, G.L., Dosemeci, M., Linet, M., Zhang, L., Xi, L., Wacholder, S., Lu, W., Meyer, K.B., Titenko-Holland, N., Stewart, J.T., Yin, S., Ross, D., 1997. Benzene poisoning, a risk factor for hematological malignancy, is associated with the NQO1 609C → T mutation and rapid fractional excretion of chlorzoxazone. Cancer Res. 57, 2839–2842.

Siegel, D., Bolton, E.M., Burr, J.A., Liebler, D.C., Ross, D., 1997. The reduction of alpha-tocopherolquinone by human NAD(P)H: quinone oxidoreductase: the role of alpha-tocopherolhydroquinone as a cellular antioxidant. Mol. Pharmacol. 52, 300–305.

Siess, M.H., Le Bon, A.M., Canivenc-Lavier, M.C., Suschetet, M., 1997. Modification of hepatic drug-metabolizing enzymes in rats treated with alkyl sulfides. Cancer Lett. 120 (2), 195–201.

Smith, M.T., Wang, Y., Kane, E., Rollinson, S., Wiemels, J.L., Roman, E., Roddam, P., Cartwright, R., Morgan, G., 2001. Low NAD(P)H: quinone oxidoreductase 1 activity is associated with increased risk of acute leukemia in adults. Blood 97, 1422–1426.

Sparnins, V., Barany, G., Wattenberg, L., 1988. Effects of organosulfur compounds from garlic and onions on benzo[*a*]pyrene-induced neoplasia and glutathione S-transferase activity in the mouse. Carcinogenesis 9 (1), 131–134.

Sumiyoshi, H., Wargovich, M., 1990. Chemoprevention of 1,2-dimethylhydrazine-induced colon cancer in mice by naturally occurring organosulfur compounds. Cancer Res. 50 (16), 5084–5087.

Talalay, P., 1989. Mechanisms of induction of enzymes that protect against chemical carcinogenesis. Adv. Enzyme. Regul. 28, 237–250.

Talalay, P., 1992. The role of enzyme induction in protection against carcinogenesis. In: Kellof, G.J. (Ed.), Cancer Chemoprevention. CRC Press, Boca Raton, FL, pp. 469–478.

Talalay, P., 2000. Chemoprotection against cancer by induction of Phase 2 enzymes. Biofactors 12, 5–11.

Vanhaecke, T., Derde, M.-P., Vercruysse, A., Rogiers, V., 2001. Hydroxypropyl-beta-cyclodextrin as delivery system for thyroid hormones, regulating glutathione S-transferase expression in rat hepatocyte co-culture. Biochem. Pharmacol. 61, 1073–1078.

Xiao, H., Parkin, K., 2006. Isolation and identification of phase II enzyme-inducing agents from nonpolar extracts of green onion (*Allium* spp.). J. Agric. Food Chem. 54 (22), 8417–8424.