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Comparative study between NAD(P)H:quinone Oxidoreductase 1 (NQO1) and Heme Oxygenase-1 (HO-1) enzymes induced by an equal dose of different classes of dietary chemicals in mice liver

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Abstract Objective: Dietary chemicals, which are generally found in vegetables, fruits and spices, have been reported to protect against cancer based on their ability to activate endogenous defenses by inducing the expression of phase II detoxification and antioxidant enzymes such as NAD(P)H:quinone Oxidoreductase 1 (NQO1) and Heme Oxygenase-1 (HO-1). The objective of this study is to compare the potency of induction of NQO1 and HO-1 genes, and their protein products in mice livers following administration of equal doses (50 mg/kg) of five different dietary chemicals (sulforaphane, quercetin, curcumin, butylated hydroxyanisole, indole-3-carbinol). **Methods:** Adult male ICR white mice were divided into 8 groups (n=6 per group) i.e. normal control, sulforaphane, quercetin, curcumin, butylated hydroxyanisole, indole-3-carbinol, vehicle 1 control and vehicle 2 control groups. The chemicals were administered intraperitoneally for 14 days at a dose of 50 mg/kg body weight. At day 15, the mice were sacrificed and their livers isolated. Total RNA was extracted, reverse transcribed and subjected to quantitative real-time PCR to detect NQO1 and HO-1 gene expression. Western blots were also performed to determine NQO1 and HO-1 protein expression. **Results:** The comparison between NQO1 and HO-1 gene expression showed that equal doses (50mg/kg) of dietary chemicals induced HO-1 gene expression more potently than NQO1. Meanwhile, they induced NQO1 and HO-1 protein with the similar potency. **Conclusions:** Equal doses (50mg/kg) of sulforaphane, indole 3 carbinol, butylated hydroxyanisole and curcumin induced HO-1 gene expression more potently than NQO1 while they induced NQO1 and HO-1 protein expression with the similar potency.
Keywords: NAD(P)H: quinone oxidoreductase 1, Heme oxygenase-1, Gene expression, Protein expression, Sulforaphane, Quercetin, Curcumin, Indole-3-carbinol, Butylated hydroxyanisole, Mice, Liver.

دراسة مقارنة بين إنزيمات NQO1 و HO-1 المستحثة بجرعة متساوية من فئات مختلفة من المواد الكيميائية الغذائية في كبد الفئران

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الملخص المواد الكيميائية الغذائية التي توجد بشكل عام في الخضار والفواكه والتوابل لها القدرة على الحماية من السرطان بناء على قدرتها على تنشيط الدفاعات الذاتية عن طريق حث التعبير عن المرحلة الثانية لإنزيمات إزالة السموم والإنزيمات المضادة للأكسدة مثل NQO1 و HO-1 و لذلك فإن الهدف من هذه الدراسة هو مقارنة فاعلية حث الجينات ومنتجات البروتين لـ NQO1 و HO-1 في كبد الفئران بعد إعطاء جرعة متساوية (50 مجم / كجم) من خمس مواد كيميائية غذائية مختلفة (Sulforaphan،Quercetin ، Curcumin، Butylated hydroxyanisole،Indole-3-carbinol). أظهرت النتائج أن المقارنة بين التعبير الجيني NQO1 و HO-1 للجرعة المتساوية (50 ملجم / كجم) من المواد الكيميائية الغذائية حثت التعبير الجيني لـ HO-1 أكثر من NQO1 وفي الوقت نفسه ، تسببت هذه المواد في حث بروتينات NQO1و HO-1 بنفس القوة. نستنتج من هذه الدراسة أن المواد الكيميائية الغذائية المختبرة بجرعة متساوية استطاعت حث التعبير الجيني لإنزيم HO-1 أكثر من NQO1 ، بينما تسببت هذه المواد في حث بروتينات NQO1 و HO-1 بنفس القوة.
الكلمات المفتاحية: NQO1،HO-1، تعبير الجين، تعبير البروتين، Sulforaphane، Quercetin، Curcumin، Indole-3-carbinol، Butylated hydroxyanisole، الفئران، الكبد.

Introduction

Epidemiological studies have suggested that regular consumption of fruits, vegetables and

spices helps in preventing various types of cancer [1-6]. These anti-cancer activities have been, at least in part, attributed to the anti-oxidant properties [7]. These dietary chemicals promote the removal of reactive oxygen species generated during normal oxidative metabolism and by reactions initiated by unwanted xenobiotic chemicals [8]. A wide variety of dietary polyphenols and other classes of dietary chemicals have been reported to induce the expression of enzymes involved in both cellular antioxidant defenses and elimination/inactivation of electrophilic carcinogens [9]. Some of these proteins are classified as phase II drug metabolizing enzymes, although other enzymes and antioxidant proteins are also involved. These proteins are known as phase II proteins [10]. Induction of such cytoprotective enzymes by dietary chemicals is recognized as one of the highly effective strategies for preventing cancer in the human population [11]. Phase II proteins have been recognized to be upregulated by the ARE/Nrf2 anti-oxidative signaling pathway [12-14]. NQO1 and HO-1 are examples of Phase II proteins regulated by Nrf2 [15, 16].

NAD(P)H: quinone oxidoreductase 1 (NQO1), a cytosolic protein of the flavoprotein clan, functions as part of the electrophilic and/or oxidative stress-induced cellular defense mechanism and reduces and detoxifies toxic quinones and semiquinones generated via the phase I mechanisms thereby preventing formation of DNA adducts by these toxic intermediates [17-19].

Heme oxygenase-1 (HO-1) catalyzes the first rate-limiting step in the catabolism of the pro- oxidant heme to carbon monoxide, biliverdin, and free iron [20]. HO-1 can have both anti- oxidative and anti-inflammatory effects, as biliverdin can be reduced to the antioxidant bilirubin, by biliverdin reductase, and small amounts of carbon monoxide can have anti-inflammatory effects [21].

Many dietary chemicals bolster intrinsic antioxidant defenses in cells, including induction of expression of antioxidant enzymes such as heme oxygenase (HO), and others as well as redox enzymes such as NAD(P)H quinone oxidoreductase 1 (NQO1) [22]. However, no one has ever compared which enzyme is the most potent following administration of equal doses of dietary chemicals in mice liver where the liver is healthy and not exposure to any disease and toxicity. Therefore, the novelty of this current study lies on the identification of the most potent enzyme induced by the dietary chemicals studied.

Therefore, this study is to compare the potency of induction of NQO1 and HO-1 genes, and their protein products in mice livers after administration of an equal dose (50 mg/kg) of five different dietary chemicals (sulforaphane, quercetin, curcumin, butylated hydroxyanisole, indole-3- carbinol).

Materials and Methods

Chemicals and reagents

Primers were purchased from Vivantis Technologies (Oceanside, CA, USA). TRIzol Reagent was purchased from Life Technologies (Carlsbad, California, USA). iScript™ cDNA Synthesis kit and iQTM SYBR® Green Supermix (2X) were purchased from Bio-Rad (Hercules, California, USA). Sulforaphane was purchased from Santa Cruz Biotechnology (Paso Robles, California, USA). NQO1 rabbit polyclonal primary antibody, HO-1 polyclonal primary antibody and β-actin rabbit polyclonal primary antibody were purchased from Abcam Biotechnology (Cambridge, UK). Secondary antibody (goat anti-rabbit IgG) were purchased from Santa Cruz Biotechnology (Paso Robles, California, USA. Chemiluminescence Western blotting detection reagents were from GE Healthcare (Uppsala, Sweden). Nitrocellulose membrane and Ponceau S solution were purchased from Sigma-Aldrich (Seelze, Germany). Sulforaphane was purcased from Santa Cruz Biotechnology (Paso Robles, California, USA). Curcumin, quercetin, I3C, BHA and all other chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

Animals used and their treatments

48 adult Male ICR white mice (25-30g) were obtained from the Universiti Kebangsaan Malaysia Laboratory Animal Research Unit and were used in this study. The mice were kept in clean polypropylene cages in a ventilated room with a 12-hour light-dark cycle, with food and water available *ad libitum*. Mice were divided into 8 groups: normal control group (n=6), sulforaphane treated group (n=6), quercetin treated group (n=6), curcumin treated group (n=6), BHA treated group (n=6), I3C treated group (n=6), vehicle 1 control group (n=6) and vehicle 2 control group (n=6). All chemicals were administered intraperitoneally at a dose of 50 mg/kg body weight for 14 days. Vehicle 1 (DMSO, Tween 20 and normal saline at a ratio of 0.05:0.1:0.85) was used to dissolve sulforaphane, quercetin and curcumin. Vehicle 2 (corn oil) was used to dissolve BHA and I3C. At day 15, the animals were sacrificed by cervical dislocation and their livers isolated. The procedures of studying the animals was confirmed by the University of Kebangsaan Malaysia Animal Ethics Committee (UKMAEC), and the approval code was: FP/FAR/2012/AZMAN/23- MAY/442-JUNE-2012-JUNE-2015.

RNA Extraction

Total RNA from frozen liver tissues was isolated using TRIzol reagent, based on the instructions provided by the manufacturer. Isopropyl alcohol (Sigma, USA) was added during each extraction step to precipitate the total RNA. 75% ethanol was used to wash the extracted total RNA pellet, then the pellet was dried before being dissolved in RNase free water. Total RNA was stored at -80 °C immediately after extraction. Concentration and purity of the extracted RNA were determined by NanoDrop spectrophotometer 2000c (Thermo Scientific, USA) at a wavelength of 260 nm (OD260). RNA with RNA integrity number (RIN) ranging from 7 to 10 and absorbance ratio of A260 to A280 ranging from 1.5 to 2.0 was used for cDNA synthesis.

Reverse transcription

Generation of cDNA from RNA was done using

iScript cDNA synthesis kit (Bio-Rad, USA) according to the manufacturer's instructions. Briefly, in each sample, a volume of total RNA (containing 1 µg) was added to the mixture of 4 µl of 5X iScript reaction mix, 1 µl of iScript reverse transcriptase, and a volume of nuclease-free water in a total volume of 20 µl. The final reaction mix was kept at 25 °C for 5 min, 42 °C for 30 min, and heated to 85 °C for 5 min in a thermocycler (TC-412, Techne, Barloworld Scientific, UK). The cDNA was then used as a template for amplification by polymerase chain reaction (PCR).

Quantification of NQO1 and HO-1 gene expression by quantitative real-time PCR

Quantitative real-time PCR was performed on the MiniOpticon cycler (Bio-Rad, USA). The total reaction volume used was 20 µl, consisting of 1 µl of 10 µM forward primer and 1 µl of 10 µM reverse primer (500 nM final concentration of each primer), 10.0 µl of iQ™ SYBR® Green Supermix (2X) (Bio-Rad, USA), 6.0 µl of nuclease-free water and 2.0 µl of cDNA. Both forward and reverse primers for the genes of interest in this study were designed according to previous studies and synthesized by Vivantis Technologies (Oceanside, CA, USA). The primer sequences for our genes of interest are shown in Table 1.

The thermocycling conditions were initiated at 95°C for 30 sec, followed by 40 PCR cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 30 sec. At the end of each cycle, a melting curve (dissociation stage) was performed in order to determine the specificity of the primers and the purity of the final PCR product. All the measurements were performed in triplicate and no-template controls (NTC) were incorporated onto the same set of PCR tubes to test for the contamination by any assay reagents. Threshold cycles were determined for each gene and quantification of templates was performed according to the relative standard curve method. The relative gene expression (ΔΔCt) technique, as defined in the Applied Biosystems User Bulletin No. 2 [23] was used to analyse the real-time PCR data. In short, the expression level of each target gene was given as relative amount normalized against GAPDH standard controls.

Table 1. Primer sequence for GAPDH, NQO1 and HO-1.

Gene	Primer sequence	Reference
GADPH	F: 5′ -GTGGAGTCTACTGGTGTCTTCA-3′ R: 5′ -TTGCTGACAATCTTGAGTGAGT-3′	[24]
NQO1	F: 5′ -GCATTGGCCACAATCCACCAG-3′ R: 5′ -ATGGCCCACAGAGAGGCCAAA-3′	[25]
HO-1	F: 5′ -CCTCACTGGCAGGAAATCATC-3′ R: 5′ -TATGTAAAGCGTCTCCACGAGG-3′	[26]

Preparation of cytosolic protein fraction

Liver tissue samples were homogenized in RIPA lysis buffer (which contained 10 µl phenylmethylsulfonyl fluoride (PMSF), 10 µl sodium orthovanadate and 10 µl protease inhibitor cocktail solution per 1 ml of 1X RIPA lysis buffer). After centrifugation, the supernatants were collected and their protein concentrations were determined by the Lowry method using bovine serum albumin as a standard [27].

Western blotting

Standard Western blotting procedure was used for the immunodetection of proteins. Briefly, 100 µg of liver protein was separated using 15% sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE) electrophoresis. The proteins in the gel were then transferred to a nitrocellulose membrane. The membrane was then incubated for 20 minutes at room temperature in a blocking solution (150 mM NaCl, 3 mM KCl, 25 mM Tris, 0.1% (v/v) tween- 20 and 10% non-fat milk powder (pH 7.4)). Then, the membrane was probed with primary antibodies against HO-1, NQO1, and β-actin for 1 hour at room temperature. Subsequently, incubation with a peroxidase conjugated goat anti-rabbit IgG secondary antibody was carried out for another hour at room temperature. Protein bands were visualized using the enhanced chemiluminescence method according to the manufacturer's instructions (Amersham, Uppsala, Sweden). The intensity of the protein bands was quantified, relative to the signals obtained for actin, using ImageJ software.

Statistical Analysis

At least three independent experiments were conducted for each analysis. Statistical analysis was performed by the Student's two-tailed unpaired t-test. All values are expressed as the mean ± standard error of the mean (SEM). SPSS for Windows, version 22.0 (SPSS, Chicago, IL, USA), was used for all statistical analyses, and statistical significance was accepted at p-values < 0.05.

Results

Comparison between NQO1 and HO-1 gene expression

The comparison between the expression levels of NQO1 and HO-1 gene in mice liver following administration of 50mg/kg SUL, CUR, QRC, BHA and I3C for 14 days in mice is shown in Figure 1. NQO1 and HO-1 gene expression in the liver was measured using quantitative real-time PCR. Administration of 50 mg/kg SUL and BHA caused a significant increase in HO-1 gene expression levels compared to NQO1 ($P<0.05$). On the other hand, Administration of 50 mg/kg CUR and I3C caused a significant increase in HO-1 gene expression levels compared to NQO1 ($P<0.01$). In the quercetin treated group, NQO1 gene expression levels was found to be statistically insignificant when compared with HO-1 gene expression.

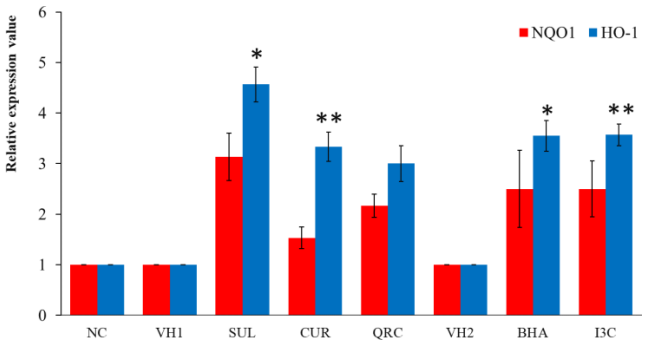

Figure1: Comparison between NQO1 & HO-1 genes expression in mice livers following administration of 50mg/kg SUL, CUR, QRC, BHA and I3C for 14 days. Student's t-test * and **denote significantly different at p< 0.05 and p <0.01respectively.

Comparison between NQO1 and HO-1 protein expression

The comparison between the expression levels of NQO1 protein and HO-1 protein in mice liver following administration of 50mg/kg SUL, CUR, QRC, BHA and I3C for 14 days in mice is shown in Figure 2. NQO1 and HO-1 protein expression in the liver was measured using Western blot.

The comparison between the expression levels of NQO1 and HO-1 proteins in mice liver following administration of 50mg/kg SUL, CUR, QRC, BHA and I3C for 14 days was not statistically significant.

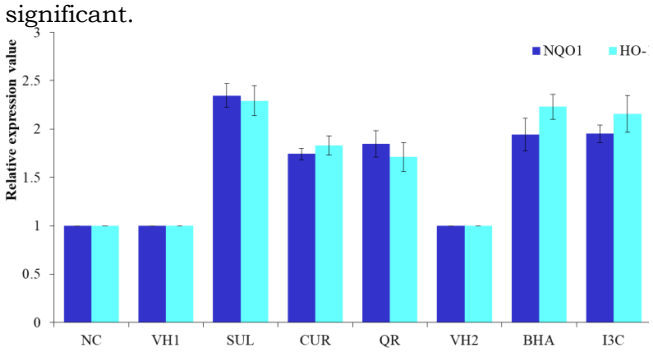

Figure2: Comparison between NQO1 & HO-1 proteins expression in mice livers following administration of 50mg/kg SUL, CUR, QRC, BHA and I3C for 14 days.

Discussion

In this study, we examined the expression of NQO1 and HO-1 genes and their proteins in the livers of mice treated with equal dose (50 mg/kg body weight) of sulforaphane, curcumin, quercetin, butylated hydroxyanisole and indole-3-carbinol for 14 days using quantitative real- PCR and Western blotting.

In our previous study, we found that all five dietary chemicals (sulforaphane, curcumin, quercetin, butylated hydroxyanisole and indole-3-carbinol) produced a significant induction of NQO1 [28] and HO-1 expression genes [29, 30], and their protein products in mice livers [28, 30].

In the current study we compared the effect of equal doses (50mg/kg) of sulforaphane, indole 3 carbinol, butylated hydroxyanisole, curcumin and quercetin on NQO1 with that of the HO-1 genes expression in mice livers.

As illustrated in Figure 1, the comparison between NQO1 and HO-1 gene expression showed that equal doses (50mg/kg) of sulforaphane, indole 3 carbinol, butylated hydroxyanisole and curcumin induced HO-1 gene expression more potently than NQO1 while NQO1 gene expression levels induced by quercetin was found to be statistically insignificant when compared with HO- 1 gene expression.

Figure 2 showed that the comparison between NQO1 and HO-1 protein expression showed that equal doses (50mg/kg) of sulforaphane, indole 3 carbinol, butylated hydroxyanisole, curcumin and quercetin induced NQO1 and HO-1 protein with the similar potency.

Phase II along with antioxidant enzymes are responsible for the detoxification and elimination of xenobiotics and reactive metabolites including ROS that may cause damage to cells and tissues [31, 32]. The induction of these enzymes is now recognized as an efficient approach to prevent carcinogenesis [33, 34]. The key role in the induction of expression of phase II detoxifying enzymes as well as other cytoprotective proteins is played by the Nrf2–ARE pathway [35-37].

HO-1, meanwhile, is the primary rate-limiting enzyme in heme catabolism and is well known to have a cytoprotection effect against liver damage by restraining oxidative stress and inflammation. HO-1 is also involved in maintaining the oxidants/antioxidants balance [38] by increased formation of the antioxidant, bilirubin [39]. The products of HO-1 activity are now commonly regarded as protective effector molecules. It is well established that HO-1 expression protects cells from physical, chemical and biologic stress [40]. HO-1 is transcriptionally upregulated by a large variety of stimuli, e.g. heme, oxidative stress, signaling proteins and organic chemicals.

Many studies have been demonstrated that sulforaphane, curcumin, quercetin, indole-3-carbinol and butylated hydroxyanisole induced NQO1 and HO-1 genes and proteins expression in several types of cells and organs including liver cells as well as liver itself [41-49].

As illustrated in Figure 1, these dietary chemicals induced HO-1 gene expression more potently than NQO1.

There might be an explanation for these contrasting results. ARE-dependent genes may be induced by different Nrf2-containing ARE binding complexes, depending on the nature of the ARE sequence and the inducer chemical.

Previous studies reported that ARE-dependent genes may be induced by different Nrf2-containing ARE binding complexes, depending on the nature of the ARE sequence and the inducer chemical [50-52].

The –N=C=S group of most isothiocyanates is electrophilic and can react readily with various nucleophiles, including thiols [53]. Therefore, cysteine thiols in Keap1 are able to react with electrophilic group (isothiocyanates) of sulforaphane [54, 55]. Then, Nrf2 has the chance to escape from Keap1 and subsequently transfers to nuclei, and binds to Maf protein, by which

further triggers antioxidant response element (ARE). Finally, ARE activation inducing a battery of antioxidant genes, including NAD(P)H: quinone oxidoreductase 1 (NQO1) and heme oxygenase- 1 (HO-1).

Butylated hydroxyanisole (BHA) is a phenolic antioxidant which experiences O-dealkylation by cytochrome P450 isozymes to produce tert-butylhydroquinone (TBHQ) which further breaks down into to tert-butylbenzoquinone (TBQ) and these metabolites are related to reactive oxygen species (ROS) generation. However, BHA is not capable of activating oxygen. Moreover, several evidences suggest that TBHQ derived from BHA can activate Nrf2. Nrf2 activation and the subsequent up-regulation of its downstream genes during TBHQ exposure are thought to be attributable to oxidative stress (e.g., ROS formation). Previous studies reported that TBHQ itself does not activate Nrf2. This is the oxidation product TBQ, which is the ultimate inducer because of its electrophilic properties [56, 57].

Curcumin has two α,β-unsaturated carbonyl groups and can hence act as a Michael reaction acceptor, causing thiol modification of Keap1, followed by Nrf2 nuclear translocation. Furthermore, polyphenolic compounds that contain α,β-unsaturated ketone element are reported to function as potent Michael-reaction acceptors and modify the cysteine and thiols groups in Keap1 thereby disrupting Nrf2-Keap1 interactions [58, 59].

There are clear distinctions between the potencies of different classes of inducers and the chemistry of interaction with Keap1. Moreover, the fact has been established that they each display specific off target responses, presumably exhibiting their chemical mechanisms and propensities for reacting with different sulfhydryl regulated signaling pathways within a cell.

Following the aforementioned discussion, a panel of small molecules, mainly involving compounds belonging to different structural classes: α-β unsaturated carbonyls, isothiocyanates, flavonoid, and polyphenol, with oxidative or electrophilic properties capable of modifying free thiol groups for activation of Nrf2, the master regulator of the oxidative stress response [15, 60].

Results from qRT-PCR showed that dietary chemicals upregulated a battery of NQO1 and HO-1 genes, indicating that dietary chemicals triggered Nrf2-ARE pathway. As a cytoplasmic Nrf2- interacting protein, Keap1 negatively regulates Nrf2 activity. Dissociating the complex of Keap1- Nrf2 is appeared as a good strategy to activate Nrf2. There are other mechanisms of Nrf2 activation, such as Keap1 cysteine-independent.

These dietary chemicals might activate MAPK/PKC pathways in different ways, all of which could affect ARE activation at various intensities or levels.

A number of studies have revealed alternative mechanisms of Nrf2 regulation, including phosphorylation of Nrf2 by various protein kinases (PKC, PI3K/Akt, GSK-3b, JNK)[61-63], interaction with other protein partners (p21[64], p62(sequestosome 1) [65] and caveolin-1) and epigenetic factors (micro-RNAs -144, -28 and -200a, and promoter methylation) [66].

There are some limitations associated with this study. The Nrf2-ARE system has been reported to be modulated by different MAPKs, interaction with other protein partners (p21, p62) and epigenetic factors (micro-RNAs -144, -28 and -200a, and promoter methylation). These factors are not explored in this study due to their highly controversial nature because modulation might occur through indirect mechanisms with limited effects.

Conclusion

According to the findings of this study, equal doses (50mg/kg) of sulforaphane, indole 3 carbinol, butylated hydroxyanisole and curcumin induced HO-1 gene expression more potently than NQO1. Equal doses (50mg/kg) of sulforaphane, indole 3 carbinol, butylated hydroxyanisole, curcumin and quercetin induced NQO1 and HO-1 protein with the similar potency. Increases in HO-1 expression has clear anti-inflammatory and antioxidant effects and can protect tissues and organs. Therefore, it is possible to predict that dietary chemicals can exhibit its pharmacological properties in liver tissue. It has been proposed that HO-1 induction by pharmacological compounds contributes to at least some of the perceived therapeutic effects. This work provides novel sights in comprehending the underlying of the liver protection and also sheds lights on the development of new drugs for the attenuation of liver diseases progression. Therefore, consumption of dietary chemicals could potentially prove to be beneficial in preventing liver toxicity and could also potentially be a cost-effective cancer chemoprevention measure.

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