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Xanthohumol Isolated from *Humulus lupulus* Inhibits Menadione-Induced DNA Damage through Induction of Quinone Reductase

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Abstract

The female parts of hops (*Humulus lupulus* L.) show estrogenic effects as well as cancer chemopreventive potential. We analyzed the chemopreventive mechanism of hops by studying its antioxidative activities and its effect on the detoxification of a potentially toxic quinone (menadione). The detoxification enzyme quinone reductase [(NAD(P)H:quinone oxidoreductase, QR] protects against quinone-induced toxicity and has been used as a marker in cancer chemoprevention studies. Although the hop extract was only a weak quencher of free radicals formed from 1,1-diphenyl-2-picrylhydrazyl, it demonstrated strong QR induction in Hepa 1c1c7 cells. In addition, compounds isolated from hops including xanthohumol (XH) and 8-prenylnaringenin were tested for QR induction. Among these, XH was the most effective at inducing QR with a concentration required to double the specific activity of QR (CD value) of 1.7 ± 0.7 μM. In addition, pretreatment of Hepa1c1c7 cells with XH significantly inhibited menadione-induced DNA single-strand breaks. The QR inhibitor dicumarol reversed the protective effect of XH against menadione-induced DNA damage. Because the expression of QR and other detoxifying enzymes is known to be upregulated by binding of the transcription factor Nrf2 to the antioxidant response element (ARE), the reporter activity mediated by ARE in HepG2- ARE-C8 cells was investigated after incubation with XH for 24 h. Under these conditions, XH increased ARE reporter activity in a dose-dependent manner. One mechanism by which XH might induce QR could be through interaction with Keap1, which sequesters Nrf2 in the cytoplasm, so that it cannot activate the ARE. Using LC-MS-MS, we demonstrated that XH alkylates human Keap1 protein, most likely on a subset of the 27 cysteines of Keap1. This suggests that XH induces QR by covalently modifying the Keap1 protein. Therefore, XH and hops dietary supplements might function as chemopreventive agents, through induction of detoxification enzymes such as QR.

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Introduction

The strobili of hops (*Humulus lupulus* L., Cannabaceae) have long been used as a flavoring agent in beer and also as a dietary supplement for mood and sleep disturbances (1). Recently, the estrogenic activity of hops was demonstrated by a variety of in vitro and in vivo assays (1-4). Accordingly, the use of hop extracts for the treatment of menopausal symptoms is under intense investigation (5). In addition, compounds from hops interfere with different stages of carcinogenesis (6-8), and there is evidence suggesting that hops has a significant chemopreventive potential (1, 8). However, the mechanism by which hops exerts its chemopreventive activity has not been investigated in any detail.

The primary prevention of cancer is one key method of controlling the disease (9). Two main cancer preventive mechanisms are the inhibition of formation and/or detoxification of carcinogens by chemical or natural agents (10-13). The generation of reactive oxygen species (ROS)[1] by some cytotoxins can be inhibited by antioxidants such as polyphenols through their radical scavenging activities (14). Because botanical dietary supplements, such as black cohosh or green tea preparations, contain such radical scavengers, they also function as antioxidants (15, 16). The removal of ultimate carcinogens can be accomplished by detoxification enzymes, which eliminate reactive electrophiles by reduction and/or conjugation to make them less reactive and to enhance their excretion rates (17). As a consequence, the induction of detoxification enzymes, including quinone oxidoreductase (QR) and glutathione S-transferase (GST), is associated with decreased susceptibility to chemical carcinogens and represents an important pathway of chemoprevention (13, 18, 19). For example, sulforaphane isolated from broccoli (*Brassica* spp.) is an example of a potent inducer of detoxification enzymes that has demonstrated anticarcinogenic properties in vivo (11). In addition, various natural and synthetic chalcones and flavonoids with an α,β-unsaturated ketone function have shown QR inducing activities (19, 20).

As mentioned above, QR is an important detoxifying enzyme (19). It is a cytosolic flavoprotein that catalyzes two-electron reductions of quinones to hydroquinones, which are then susceptible to glucuronidation and excretion (Scheme 1) (21). The two-electron reduction of quinones prevents the formation of unstable semiquinones and ROS, which are generated by phase I enzymes such as cytochrome P450 reductase that only catalyze one-electron reductions of quinones (22). The unstable semiquinones undergo redox cycling in the presence of molecular oxygen generating ROS and causing oxidative stress, mutagenisis, and/or carcinogenesis through the formation of DNA single-strand breaks, mutations, crosslinks, or oxidation of DNA bases (21, 23, 24).

Prenylated flavonoids are one major class of compounds in hops (25). They are divided into two groups, prenylated chalcones with an α,β-unsaturated ketone function and prenylated flavanones. Xanthohumol (XH), the major chalcone found in hops, is also the main

[1]Abbreviations: ARE, antioxidant response element; BF, 4′-bromoflavone; CD, concentration required to double the specific activity of QR; CI, chemopreventive index; comet assay, alkaline single cell gel electrophoresis assay; CV, crystal violet; DPPH, 1,1-diphenyl-2-picrylhydrazyl; FAD, flavin adenine dinucleotide; GSH, glutathione; GST, glutathione S-transferase; MTT, 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide; NADP, nicotinamide adenine dinucleotide phosphate; qNMR, quantitative NMR; QR, NAD(P)H:quinone oxidoreductase; ROS, reactive oxygen species; XH, xanthohumol.

prenylflavonoid of hops (0.1–1% dry weight) and accounts for approximately 82–89% of all prenylated flavonoids in hops (25-27). Because various flavonoids are known to have antioxidant properties (14, 28) and because chalcones, as weak electrophiles, are known inducers of detoxification enzymes (20, 29), hop extracts should be a rich source of chemopreventive compounds.

On the basis of this information, we analyzed the chemopreventive mechanisms of hops and its polyphenolic constituents, in particular XH, in detail. The results suggest that hops and XH in particular are potent inducers of QR, which suggests that hops dietary supplements have the potential as chemopreventive agents.

Materials and Methods

Materials.

All chemicals and reagents were obtained from Fisher Scientific (Hanover Park, IL) or Sigma (St. Louis, MO), unless stated otherwise. Cell culture media and supplements were obtained from Invitrogen (Carlsbad, CA).

Plant Materials.

Spent Nugget hop pellets (plant material remaining after supercritical CO2 extraction of pelletized strobiles of *H. lupulus* cv. Nugget) were obtained from Yakima Chief (lot no. PE-MANUO04; Sunnyside, WA).

Extraction and Isolation.

An extract of Nugget hops was prepared by maceration of the plant material (642 g) in MeOH (1.5 L) overnight and removal of the marc (plant material after extraction) by gravity filtration. The chloroform subfraction was obtained as described by Chadwick et al. (30). This fraction was first fractionated by vacuum–liquid chromatography over silica gel using a petroleum ether–EtOAc–MeOH gradient. The prenylated flavonoids were isolated and characterized as described previously (30). The purity of the compounds was tested by quantitative NMR (qNMR) and LC-MS. The XH content in the chloroform subfraction was 6.19 ± 0.08% dry weight, which was quantified by HPLC using a UV detector.

Cell Culture Conditions.

Hepa 1c1c7 murine hepatoma cells were supplied by Dr. J. P. Whitlock, Jr. (Stanford University, Stanford, CA). Cells were maintained in α-minimum essential medium (MEME) supplemented with 1% penicillin–streptomycin and 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA) and incubated in 5% CO_2 at 37 °C. HepG2 cells stably transfected with antioxidant response element (ARE) luciferase reporter (HepG2-ARE-C8) were kindly provided by Dr. A. N. Tony Kong (Rutgers University, Piscataway, NJ) (31). Cells were grown in modified F-12 medium supplemented with 10% fetal bovine serum, 1.7 g/L sodium bicarbonate, 100 units/mL penicillin, and 100 μg/mL streptomycin, essential amino acids, and insulin (32).

DPPH Assay.

Reaction mixtures containing test samples (5 μL dissolved in DMSO or water) and 95 μL of a 200 μM 1,1-diphenyl-2-picrylhydrazyl (DPPH) ethanolic solution were incubated at 37 °C for 20 min in 96 well microtiter plates. The absorbance of the free radical DPPH was measured at 515 nm with an ELISA reader (Power Wave 200 Microplate Scanning Spectrophotometer, Bio-Tek Instrument, Winooski, VT), and the percent inhibition was determined by comparison with DMSO-treated control groups. All pure compounds were screened at a concentration of 200 μM, and the hop extract was screened at 200 μg/mL. Results represent the average ± SD of three determinations.

In Vitro QR Assay.

The induction of QR activity was assessed using Hepa 1c1c7 murine hepatoma cells as described previously with minor modifications (33). Briefly, Hepa lclc7 cells were seeded in 96 well plates at a density of 1.25×10^4 cells/mL in 190 μL of media. After 24 h of incubation, test samples were added to each well and the cells were incubated for an additional 48 h. The medium was decanted, and the cells were incubated at 37 °C for 10 min with 50 μL of 0.8% digitonin and 2 mM EDTA solution (pH 7.8). The plates were then agitated on an orbital shaker (100 rpm) for 10 min at room temperature, and 200 μL of reaction mixture [bovine serum albumine, 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 25 mM Tris-HCl, 0.01% Tween 20, 5 μM flavin adenine dinucleotide (FAD), 1 mM glucose-6-phosphate, 30 μM nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate dehydrogenase, and 50 μM menadione] was added to each well. After 5 min, the plates were scanned at 595 nm. The specific activity of QR was determined by measuring NADPH-dependent menadiol-mediated reduction of MTT to a blue formazan. Induction of QR activity was calculated by comparing the QR specific activity of sample-treated cells with that of solvent-treated cells. The dicumarol inhibitable rate of menadione reduction by QR was determined after treatment with XH for 48 h and exposure to dicumarol (15 μM) for 15 min prior to measuring the QR activity. CD values represent the concentration required to double QR induction. The chemopreventive index (CI) is an indexing value generated by comparing IC_{50} with CD values (IC_{50}/CD).

Cytotoxicity Assay.

Cells were plated and treated as described for the QR assay. After the cells were treated with test samples for 48 h, the medium was decanted, and 200 μL of 0.2% crystal violet (CV) solution in 2% ethanol was added. After 10 min, the plates were rinsed for 2 min with water and dried. The bound dye was solublized by incubation at 37 °C for 1 h with 200 μL of 0.5% SDS in 50% ethanol. The absorption of CV was measured at 595 nm, and the LC_{50} values were determined.

Comet Assay.

The single-cell gel electrophoresis assay was carried out according to the manufacturer's suggested procedure (Trevigen, Gaithersburg, MD) with minor modifications (15). Hepa 1c1c7 cells were plated at 1.65×10^4 cells/mL and grown for 24 h prior to treatment with

sample. After the sample had been added, the cells were incubated for 48 h to induce QR and then treated with 10 μM menadione for 30 min. Previous studies had shown that increasing concentrations of menadione caused a dose-dependent enhancement in DNA single-strand breaks reaching a maximum at 10 μM menadione and a reduction in cell viability at doses exceeding 10 μM (15). Cells were washed with PBS, harvested, and combined at 1×10^5 cells/mL with molten low-melting agarose kept at 42 °C at a ratio of 1:10 (v/v). The combined agarose and cells (50 μL) were immediately pipetted onto Comet Slides (Trevigen). The slides were then immersed in prechilled lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium lauryl sarcosinate, 1% Triton X-100, and 10% DMSO, pH 10.0) for 30 min at 4 °C in the dark. After lysis, slides were immersed in alkaline solution (10 M NaOH and 200 mM EDTA, pH > 13) for 45 min, and then, horizontal electrophoresis was performed for 30 min at 300 mA. Slides were fixed with 70% ethanol for 5 min, dried, stained with 50 μL of SYBR Green solution (1 μL of SYBR Green Stain and 10 mL of TE buffer, pH 7.5; Trevigen), and viewed under a fluorescence microscope. The DNA damage was scored from 0 (intact DNA) to 4 (completely damaged DNA with tail only). Scores were calculated using the following formula: Score = $[(N_A \times 0 + N_B \times 1 + N_C \times 2 + N_D \times 3 + N_E \times 4)/(N_A + N_B + N_C + N_D + N_E)] \times 100$. N_A, N_B, N_C, N_D, and N_E were the number of cells demonstrating different comet tails from intact DNA (N_A) through completely damaged DNA (N_E). At least 100 cells were scored per sample.

ARE-Luciferase Activity Assay.

HepG2-ARE-C8 cells were plated in six well plates at a density of 1×10^5 cells/mL and incubated overnight (32). Cells were either stimulated with different concentrations of XH or 4′-bromoflavone (BF, positive control) or with DMSO as a negative control. After 24 h of treatment, the luciferase activity was determined according to the protocol provided by the manufacturer (Promega, Madison, WI). Briefly, cells were washed with cold PBS and harvested in passive lysis buffer. After centrifugation, 20 μL of the supernatant was used for determining luciferase activity, which was measured by a luminometer (FLUOstar OPTIMA, BMG Labtechnologies, Offenburg, Germany). The luciferase activity was normalized to protein concentration using the BCA (bicinchoninic acid) protein assay (Pierce, Rockford, IL). The data were obtained from three separate experiments and expressed as fold induction over control (treated cells/DMSO treated cells ± SD).

Alkylation of Keap1 by XH.

Full-length human Keap1 protein was expressed in and purified from *Escherichia coli.* The details of the cloning, expression, and purification of Keap1 will be published elsewhere (34). Increasing concentrations of XH (0, 11, 33, 66, 166, and 500 μM) were incubated with 11 μM Keap1 for 3 h at room temperature in Tris buffer (pH 8.0). The extent of alkylation of the protein was measured using a Voyager-DE PRO (Applied Biosystems, Foster City, CA) matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer in linear mode with 3,5-dimethoxy-4-hydroxycinnamic acid (Aldrich, Milwaukee, WI) as the sample matrix. The sample was mixed with matrix solution (10 mg/mL) at a ratio of 1:1, and 0.5 μL of the resulted mixture was applied to a 192 well plate before ionization using a UV nitrogen laser.

Statistics.

Two-tailed Student's *T* test (paired data) or oneway ANOVA with Dunnett's post test was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CH, www.graphpad.com). In all cases, a *P* value < 0.05 was considered to indicate significance. Experimental values are expressed as averages ± SD.

Results

DPPH Assay.

Free radical scavenging is commonly regarded as one mechanism responsible for protecting lipids, proteins, and DNA against oxidative damage (35). The antioxidant activities of hops and the isolated hop compounds were measured according to their relative ability to scavenge DPPH free radicals. In contrast to similar experiments with black cohosh, in which fukinolic acid had an IC_{50} of 14 μM (15), the hop extract and its pure compounds did not show a significant radical scavenging activity in this assay (Table 1). A compound is considered active if it scavenges more than 50% of free DPPH radicals at a concentration of 100 μM or lower.

QR Activity of a Hop Extract and Prenylated Chalcones.

QR catalyzes the detoxification of cytotoxic quinones such as menadione (Scheme 1) (36). For this reason, QR helps protect cellular membranes, proteins, and DNA against oxidative damage. The hop extract, the isolated prenylated chalcones, and the prenylated flavanones were tested for their QR inducing activity. Compounds or extracts were deemed to be inactive if their CD values were higher than 20 μM or 20 μg/mL. All prenylated chalcones except **12** showed QR inducing activities (Figure 1, Table 1). XH (≥99.5% purity both by qNMR and LC-MS) showed the highest activity with a CD value of 1.7 ± 0.7 μM. The QR activity induced by XH was reduced to control levels by treating the XH-treated cells for 15 min with 15 μM of the QR inhibitor dicumarol. Chalcones with similar activities were **2** and **3**. The other chalcones showed less activity, ranging from CD values of 4.5–10.2 μM. None of the flavanones **13**–**17** showed QR inducing activity. Isoxanthohumol **17** was analyzed up to a concentration of 50 μM, but it did not induce QR. The lack of inducing activity of the flavanones is consistent with literature reports, which showed that flavanols and flavanones are either ineffective or weak QR inducers (37).

Cell viability was determined to correct the observed QR activity for cytotoxicity. XH had no effect on cell viability below 10 μM; however, at concentrations higher than 10 μM, the QR inducing activity was reduced due to cytotoxicity (Figures 2 and 3). Neither the extract (measured in μg/mL) nor the other chalcones showed toxicity at concentrations up to 20 μM. The IC_{50} of XH was determined to be 30.7 ± 7.6 μM (Figure 3). The CI, which is calculated as the ratio of the IC_{50} value to the CD value (IC_{50}/CD), of XH was 18, which is just slightly lower than the chemopreventive effect of the well-known QR inducing compound sulforaphane (CI, 26).

XH Inhibits Menadione-Induced DNA Damage.

The Comet assay was carried out to evaluate the protective potential of XH against menadione-induced DNA damage. Because XH caused a reduction in cell viability at doses exceeding 10 μM (Figure 3), XH was used at a concentration of 8 μM or below. Cells were treated with XH for 48 h to induce QR. Subsequently, menadione was used as a model cytotoxic quinone to induce DNA strand breaks, and at a concentration of 10 μM after 30 min, it caused DNA damage in 91% of the cells. Pretreating the cells with XH for 48 h reduced DNA single-strand breaks induced by menadione in a dose-dependent manner with an IC_{50} of 0.7 ± 0.5 μM (Figure 4).

A modified comet assay was conducted to analyze if a radical scavenging effect and not QR induction was responsible for the inhibition of DNA damage. This time the cells were treated with XH and menadione at the same time and were incubated for 30 min. In contrast to treating the cells for 48 h with XH to induce QR, no preventive effect was observed (Figure 4).

Dicumarol Prevents XH Protection against Menadione-Induced DNA Damage.

After treating the cells with XH (4 μM) for 48 h, the QR inhibitor dicumarol (15 μM) was added. DNA damage was induced 15 min later by menadione as described above. Treatment with dicumarol restored the original level of DNA damage (Figure 5).

Induction of ARE-Mediated Reporter Activity by XH.

The induction of detoxification enzymes such as QR and GST is regulated by the ARE. HepG2-ARE-C8 cells, generated from HepG2 cells stably transfected with the pARE-TI-luciferase construct (31), were treated with different concentrations of XH (0.125–8 μM), and the luciferase activity was measured. XH dose dependently induced luciferase activity and, therefore, induced ARE reporter activity (Figure 6). Significant induction was observed at XH concentrations >4 μM.

Alkylation of Keap1 by XH.

Increasing concentrations of XH were incubated with the Keap1 protein. The mass spectra (Figure 7) demonstrated that XH covalently modified the Keap1 protein. As the concentration of XH in the incubation with Keap1 was increased, the molecular weight of Keap1 increased (Figure 7A), probably through alkylation of at least 2–3 cysteine sulfhydryl residues (Figure 7B).

Recently Nikolic et al.[2] were able to show that XH reacts with thiol nucleophiles, such as glutathione (GSH) and *N*-acetyl cysteine, in a large excess (1:500). In addition, kinetic plots for the reaction of XH with GSH (1:500) were generated, which demonstrated that GSH and XH formed reversible conjugates. Their product ion tandem mass spectra were consistent with the 1,4-addition of the thiol group onto the Michael acceptor system of XH. This

[2]Nikolic, D., Chadwick, L. R., Li, Y., Calamini, B., Mesecar, A., Farnsworth, N. R., Bolton, J. L., and van Breemen, R. B. Reactivity of XH, a prenylated chalcone from hops (*H. lupulus*), toward biological nucleophiles. Unpublished data.

clearly showed that XH was capable of reacting with thiol nucleophiles such as GSH or cysteine residues in the Keap1 protein.

Discussion

Abundant in spent hops, XH has been shown recently to exhibit antiproliferative activity against human breast and ovarian cancer cell lines, inhibit oxidation of human low-density lipoprotein in vitro, and inhibit specific cytochrome P450s (6, 7, 25, 38). Moreover, XH has been found to interfere with the initiation, promotion, and progression stages of carcinogenesis (8). To further evaluate the chemopreventive activity of hops and some isolated compounds, we tested their antioxidative, QR inducing, and DNA damage preventive activities. In addition, we analyzed the mechanism of QR induction in more detail.

Although various chemopreventive activities have already been described for hops, the mechanism for this activity is not fully understood (1, 8, 38, 39). As mentioned in the Introduction, the inhibition of the action of carcinogens and their detoxification by synthetic and natural agents are two main pathways of chemoprevention (40). Black cohosh, for example, acts as an antioxidant due to its polyphenol content (15). Broccoli, on the other hand, contains isothiocyanates, which are potent inducers of detoxification enzymes (18). Because of its high prenylflavonoid content, hops has the potential to act through both pathways (Scheme 1) (7, 14). In this study, the radical scavenging activity of the chloroform subfraction of the hop extract and various isolated compounds (Figure 1) was assessed by determining the relative ability of these compounds to scavenge the stable free radical DPPH in a simple nonenzymatic assay (41). Although no significant radical quenching activity was detected for the hop extract or for any of the isolated compounds in our investigation (Table 1), another study has reported that XH can scavenge peroxyl, hydroxyl, and superoxide anion radicals (8). Several methodological differences between these experiments and our antioxidative assay (different free radicals, measurement, and study design) may be responsible for the difference in the results, and thus, they are difficult to compare.

In addition, inhibition of lipid peroxidation was demonstrated for XH (6, 27). Stevens et al. explained the mechanism of inhibition of peroxynitrite-mediated low-density lipoprotein oxidation by XH by reaction of the α,β-unsaturated keto group with superoxide and peroxynitrite anions (27). However, it should be noted that these studies were done using 1 mM XH and it is unlikely that such high concentrations of XH could be achieved from a hops dietary supplement in vivo.

To further analyze the chemopreventive activity of hops and its compounds, their QR inducing activities were investigated. As shown in Scheme 1, QR catalyzes the detoxification of potentially carcinogenic quinones by direct two electron reduction to hydroquinones (36). For this reason, induction of QR helps protect cellular membranes, proteins, and DNA against oxidative damage. QR induction was measured in mouse hepatoma Hepa1c1c7 cells, which is a very robust and sensitive cell line, having higher basal QR enzymatic activity, and more sensitive to QR inducing agents as compared to other mammalian cell lines (42). These cells have been used extensively to assess the potential

anticarcinogenic activity of natural products (9). The QR activity was determined by measuring MTT reduction to the colored formazan by menadione based on a method established by Prochaska et al. (45, 44). The measured QR activity was inhibited by dicumarol (Figure 2), which demonstrated that the reduction of menadione (therefore the reduction of MTT) was entirely due to QR. Good QR induction activities have been demonstrated for XH (Table 1) and some other prenylchalcones (8, 20).

In contrast to other subfractions of the methanol hop extract, the chloroform subfraction, rich in prenylated flavonoids, revealed good QR induction properties (CD = 3.2 μM). Therefore, it represented a good target to search for potent QR-inducing compounds. We tested the QR activity of all isolated substances (Figure 1 and Table 1) (30) and compared their QR-inducing effects. Our results confirmed the previously stated theory that prenylated chalcones are active inducers of QR (20); however, flavans and flavanols were found to be inactive, since **14**–**17** did not show inducing activity. The purity of the compounds was tested by qNMR and LC-MS. In contrast to our studies, Gehrhäuser et al. reported that isoxanthohumol **17** was less active than XH but still showed some QR induction (Table 1) (8). The experiments of Miranda et al. showed low QR activities for prenylated flavanons, such as **15**–**17**, and no QR induction for unprenylated flavanones (20). As XH is a precursor of isoxanthohumol and 8-prenylnaringenin, it is possible that these test compounds contained XH to a small degree, which might be responsible for the QR induction in these samples. XH **1** differs from isoxanthohumol **17** only in the α,β-unsaturated ketone function. The loss of this function in **17** by spontaneous cyclization of XH leads to the loss of QR activity (Table 1 and Figure 1). This demonstrated that the α,β-unsaturated keto function is an essential functional requirement for QR induction. Dinkova-Kostova et al. suggested that if some flavanones, for example, pinostrobin, exhibit inducer activity, these molecules might be only precursors and that metabolism generates the ultimate active species (37). The fact that pinostrobin was inactive in cells with defective *Ah* receptor function, which is responsible for controlling phase I enzymes, supports this assumption. Dinkova-Kostova et al. showed further on that the potency of chalcones in inducing QR paralleled their Michael reaction acceptor activity (45). As compared to nonsubstituted chalcones, the 2′-*ortho*-hydroxy group as found in XH and in most other tested chalcones dramatically enhances QR induction (29). The 4′-hydroxy group further increases QR potency in comparison to nonsubstituted chalcones (45). Consequently, the high concentration of prenylated hop chalcones with these substitution patterns tends to enhance QR induction for this botanical. XH, the main prenylated chalcone in the hop extract, showed the strongest QR inducing activity (CD = 1.7 ± 0.7 μM). This CD value is consistent with literature data (Table 1) (8, 20). The other chalcones demonstrated slightly lower QR induction properties (Table 1). Comparing the different alkylsubstituents on C-2′/3′, it becomes clear that this moiety has a large effect on the QR induction properties. Compounds with bulky moieties at C-2′/3′ show lower QR activities as observed in **10**–**12**. It is possible that the bulky residue sterically impedes the reaction of the α,β-unsaturated ketone with a nucleophilic group.

Only XH showed cytotoxicity in the CV assay (Table 1) but only at higher concentrations (Figure 3, LC_{50} = 30.7 μM). The CI of XH was determined to be 18 and is similar to that of

sulforaphane, which is a common QR inducer and chemopreventive agent (19), that has demonstrated anticarcinogenic activity in vivo (11).

Previously, XH was reported to induce QR in a monofunctional pattern (20). Monofunctional inducers selectively regulate the expression of detoxification enzymes, such as GST and QR, through the ARE. In contrast to bifunctional inducers, they do not activate the xenobiotic responsive element, which regulates aryl hydrocarbon hydroxylase activity (CYP1A1) and other phase I enzymes. Because phase I enzymes can activate procarcinogens to their ultimate reactive species, monofunctional agents that induce phase II and detoxification enzymes selectively, such as QR, are more desirable candidates for cancer chemoprevention than bifunctional inducers (46). Because XH and other chalcones only induce detoxification enzymes through the ARE, they are potentially valuable chemopreventive agents.

Using the single-cell gel electrophoresis assay (comet assay), a standard tool for the measurement of DNA damage in individual cells (47), we analyzed whether QR induction by XH could protect cellular DNA from menadione-induced DNA damage. Menadione is known to cause DNA strand cleavage mediated by free radicals and semiquinones during redox cycling (Scheme 1) (15). The extent of DNA single-strand breaks caused by menadione was assessed after a 48 h pretreatment of mouse hepatoma cells with XH or without pretreatment of XH by adding menadione and XH at the same time. A dose-dependent inhibition of DNA damage was observed after a 48 h pretreatment with XH but not without XH pretreatment (Figure 4). This suggests that an enzyme inducing effect might be responsible for the protective effect of XH and not radical scavenging properties. Further evidence for the role of QR in protecting DNA from menadione-induced DNA strand cleavage was provided in experiments with the potent QR inhibitor, dicumarol. The protective effect of XH pretreatment against menadione-induced DNA damage was eliminated in the presence of dicumarol (Figure 5). This provides evidence that the DNA protective effect of XH is mediated through the increased level of the detoxifying enzyme QR.

To study the mechanism of QR stimulation by XH, the induction of ARE, which mediates the translation of detoxifying enzymes, was determined. The ARE-mediated reporter activity was determined by measuring the luciferase activity in HepG2 cells stably transfected with an ARE-luciferase reporter (31). A dose-dependent induction of luciferase activity was observed by a 24 h treatment with XH at concentrations ranging from 0.125 to 8 μM (Figure 6). Significant induction of luciferase activity by XH was achieved by doses of 4 μM XH and higher ($P < 0.05$). The upregulation of ARE by XH can lead to the induction of numerous detoxification enzymes, including QR and GST, thus potentiating the overall chemopreventive activity of XH. Two proteins, Keap1 and Nrf2, are mainly involved in regulating the expression of detoxification enzymes and antioxidant proteins through the ARE (48) (Scheme 2). Keap1 is a cytosolic inhibitor of Nrf2, which is a transcriptional activator of ARE regulated genes (49). Under basal conditions, Nrf2 is complexed with Keap1 in the cytoplasm (48). Upon introduction of inducing agents, which can either oxidize, thiolate, or alkylate Keap1 (37), Nrf2 accumulates in the nucleus where it binds to the 5′-upstream regulatory ARE regions of detoxification genes and accelerates their

transcription (48, 50). According to Dinkova-Kostova et al. (50), the 25 sulfhydryl groups of mouse Keap1 function as the sensors for the inducers. Not only do known potent QR inducers, including sulforaphane and the Michael reaction acceptor bis(2-hydroxybenzylidene)acetone, bind to mouse Keap1, but also the ability to bind Keap1 correlates with the induction ability (50, 51). Cysteine mutagenesis studies implicate at least three Keap1 cysteines in signaling for Nrf2 nuclear accumulation. These studies show that C273 and C288 mutants constitutively upregulate the ARE due to constitutive Nrf2 nuclear accumulation (59, 60). In addition, a C151S mutant did not respond to electrophiles, and C151S was shown to be highly reactive to electrophiles indicating that Keap1 modification by electrophiles is essential for signaling (34, 61). Other reports state that the ARE is activated by quinones, compounds that undergo redox cycling, and α,β-unsaturated carbonyls (46). The electrophilic Michael reaction acceptor group of XH, the α,β-unsaturated ketone function, has been shown to react with thiol nucleophiles.[2] To test if XH reacted with the biologically relevant protein Keap1, we incubated XH with Keap1. XH modified the Keap1 protein through the alkylation of at least 2–3 sites, most likely cysteine sulfhydryl residues (Figure 7 and Scheme 2). Cumulatively, the data suggest that the α,β-unsaturated carbonyl of XH allows binding to human Keap1 and that XH might upregulate the transcription of ARE-mediated detoxifying genes by directly binding to Keap1 protein. This mechanism would also explain why the α,β-unsaturated ketone function in chalcones is essential for inducing QR (45).

In conclusion, these data show that the chloroform subfraction of spent hops with its high prenylpolyphenol content and rich in XH exhibits good chemopreventive activity through induction of QR. Therefore, it is reasonable to conclude that XH might be useful as a biologically active marker compound for the standardization of hops preparations for use as cancer preventive dietary supplements.

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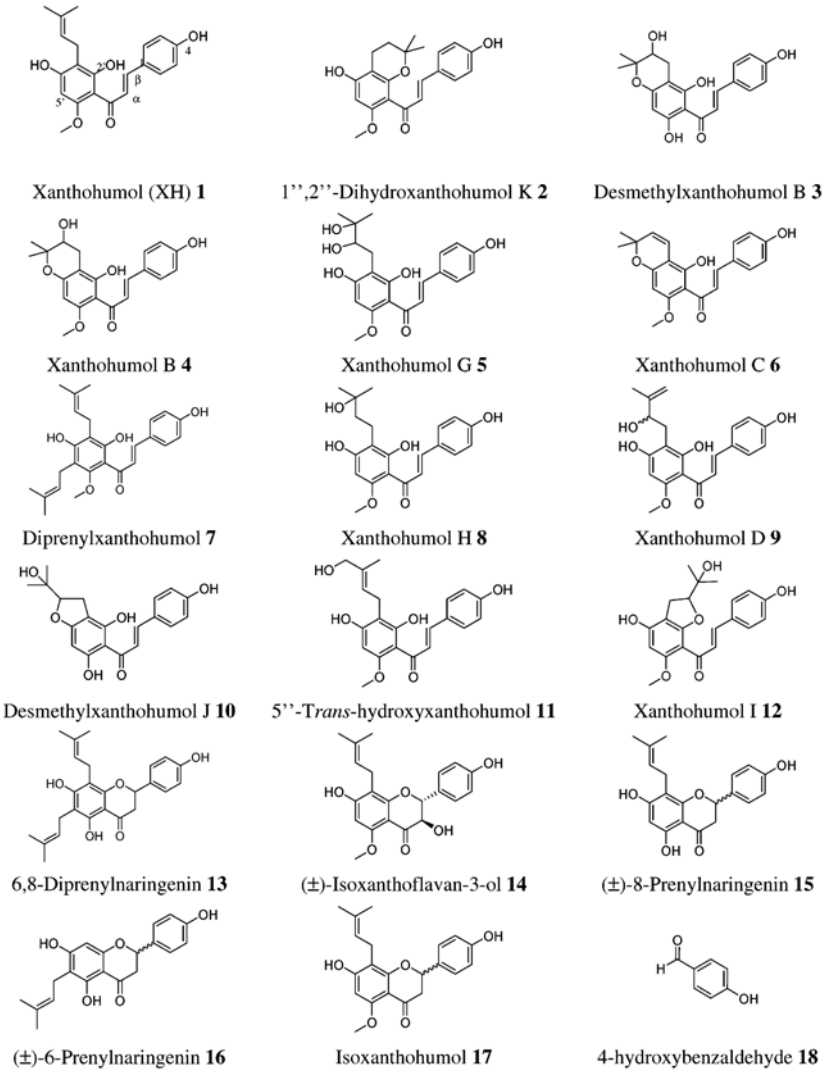


Figure 1.
Chemical structures of isolated compounds from *H. lupulus* L. (30).

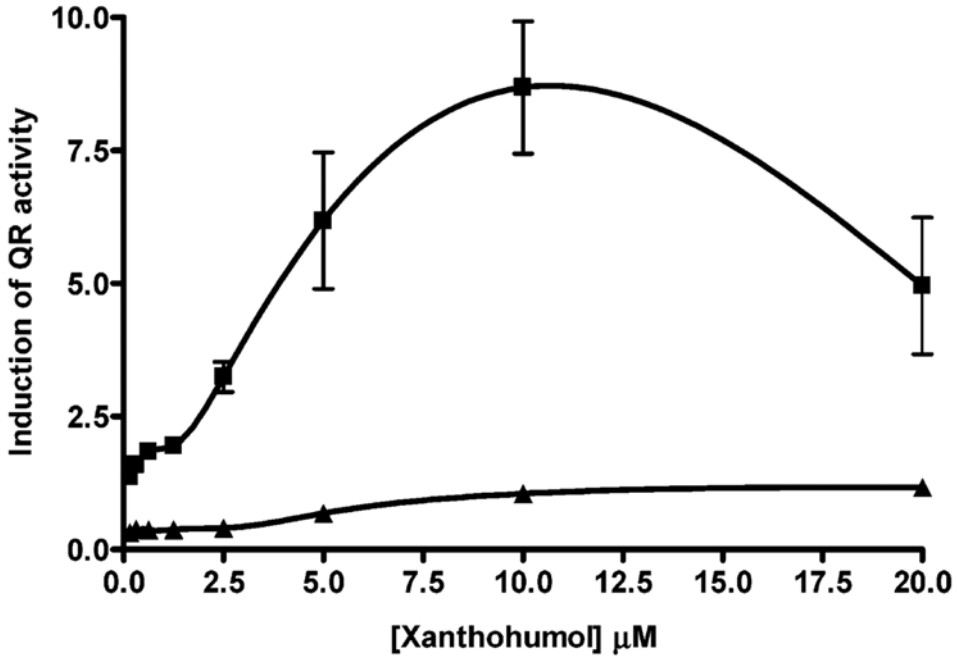


Figure 2.

QR activity of XH isolated from hops was evaluated using Hepa 1c1c7 murine hepatoma cells. After the cells were treated with XH for 48 h (■), the induction of QR was determined. In parallel, cells (▲) were preincubated with XH for 48 h prior to exposure to dicumarol (15 μM) for 15 min, and subsequently, the QR activity was measured. Each value represents the average fold induction of three experiments performed independently in duplicate ± SD.

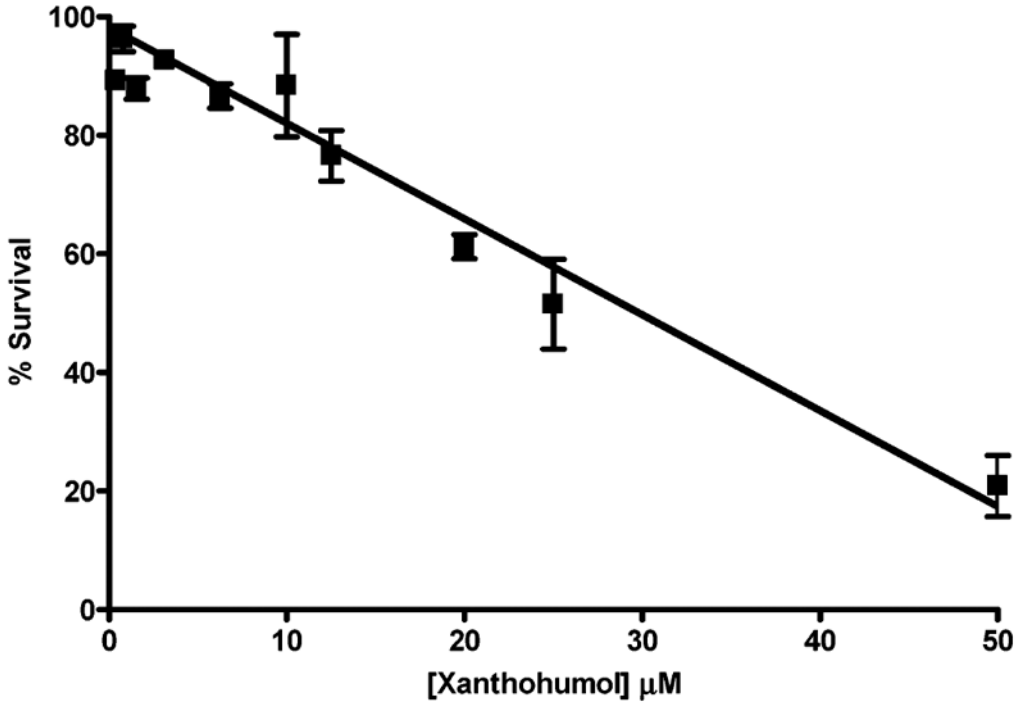


Figure 3.

Cytotoxicity of XH was examined in the CV assay. The cells were treated with XH for 48 h. Each value represents the average of three experiments performed independently in duplicate ± SD.

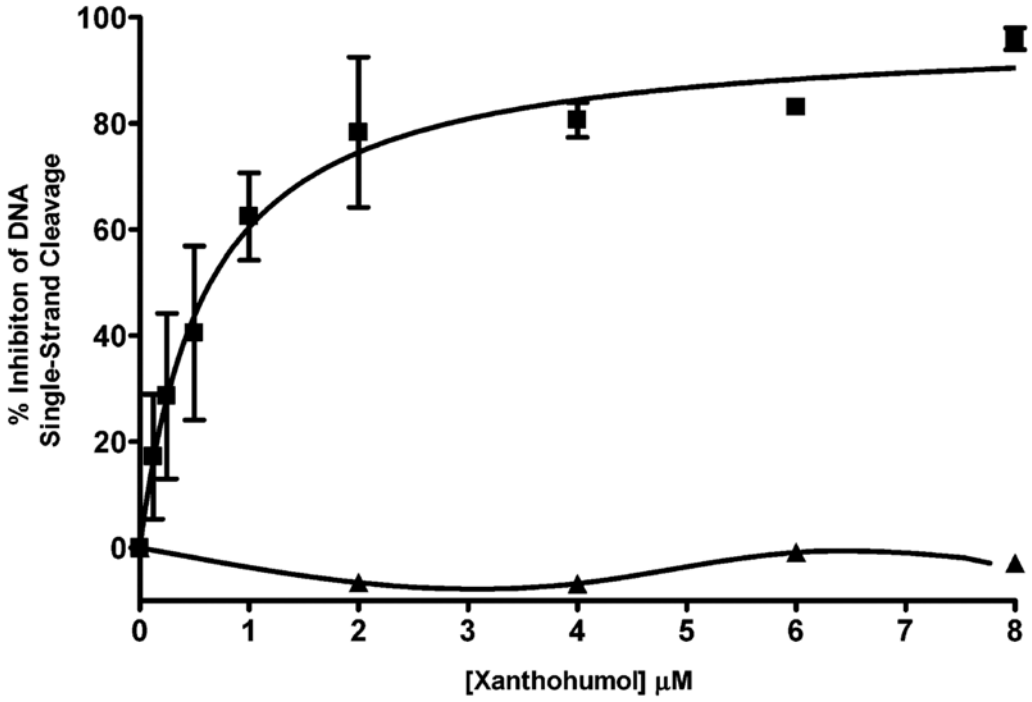


Figure 4.

Inhibitory effect of XH against DNA single-strand breaks induced by menadione was examined using the comet assay. Hepa 1c1c7 mouse hepatoma cells were preincubated with XH for 48 h prior to menadione (10 μM) exposure for 30 min (■), or cells were treated with XH and menadione at the same time for 30 min (▲) before the comet assay was conducted. Each value is the average of triplicate measurements ± SD.

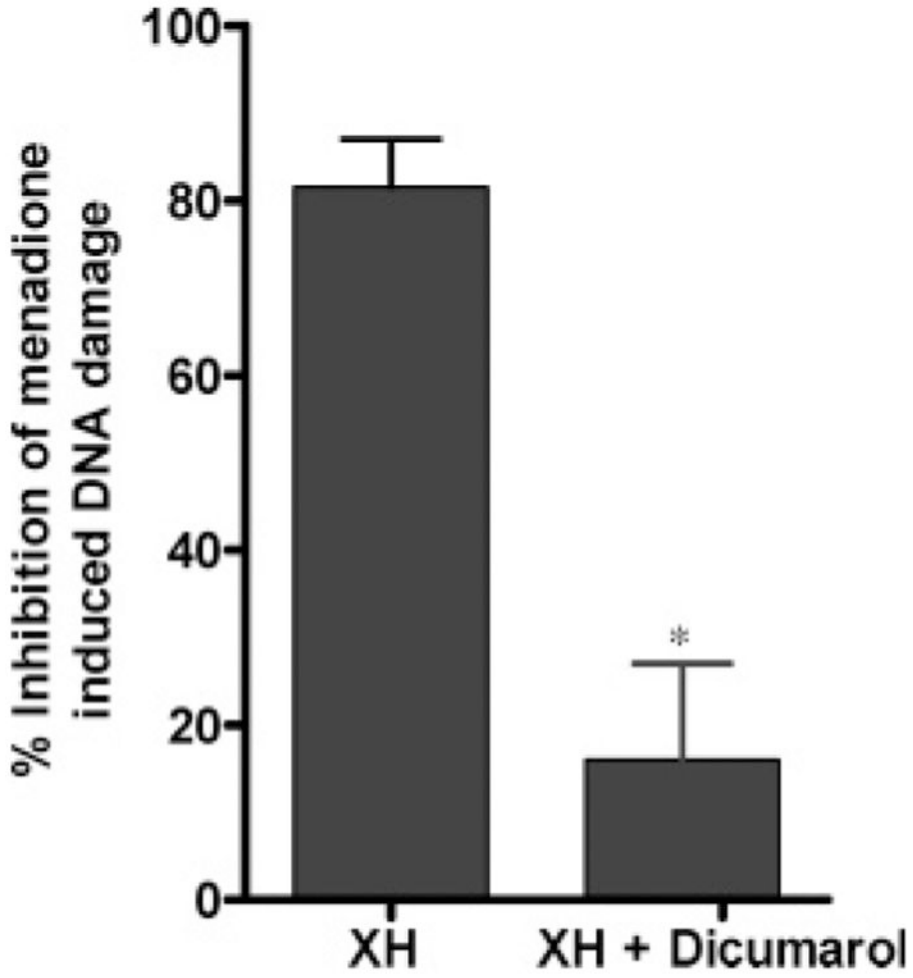


Figure 5.

Hepa 1c1c7 mouse hepatoma cells were preincubated with XH (4 μM) for 48 h prior to exposure to dicumarol (15 μM) for 15 min. DNA single-strand breaks were induced by 10 μM menadione treatment for 30 min. Each value is the average of triplicate measurements ± SD. Means are significantly different ($P < 0.05$, t-test).

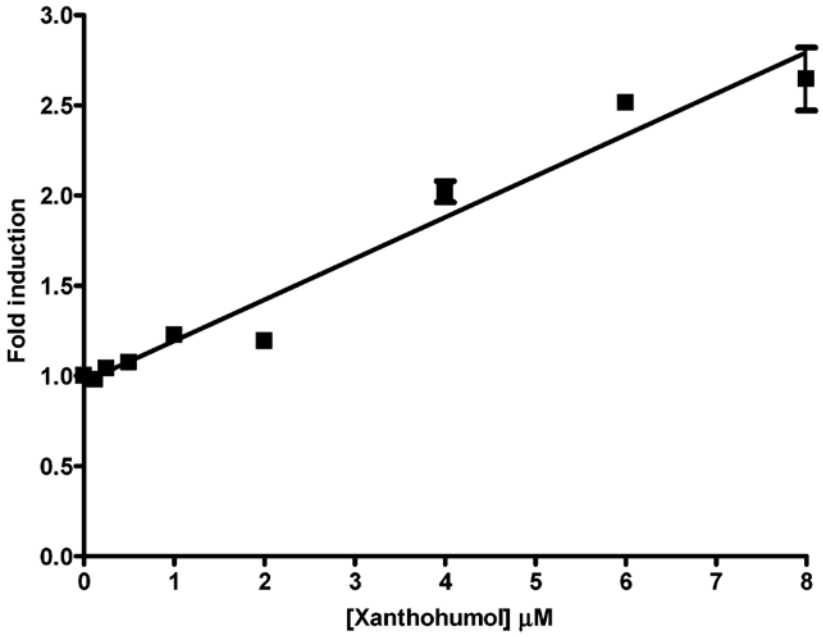


Figure 6.

Induction of the ARE-luciferase reporter gene by XH. Stably transfected HepG2-ARE-C8 cells (54) were plated in six well plates at a density of 1×10^5 cells/mL and incubated overnight. Cells were stimulated with different concentrations of XH, BF as a positive control (40 μg/mL, 6.32 ± 0.98-fold induction), or with DMSO as a negative control. Cells were harvested 24 h after treatment. Luciferase activity was determined and normalized by protein determination. The data were obtained from three separate experiments and expressed as fold induction as compared to the control (DMSO-treated cells) ± SD.

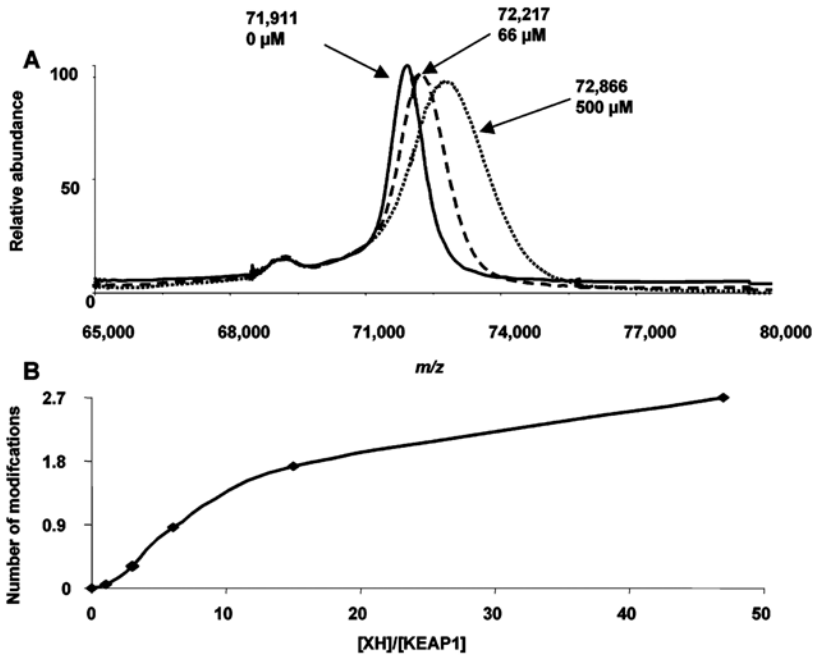
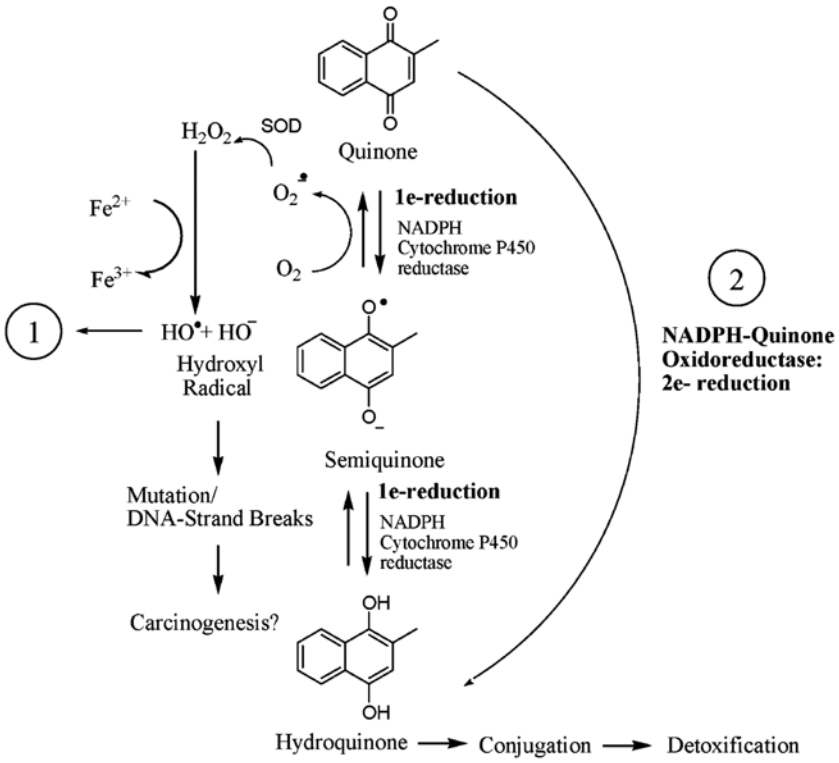


Figure 7.

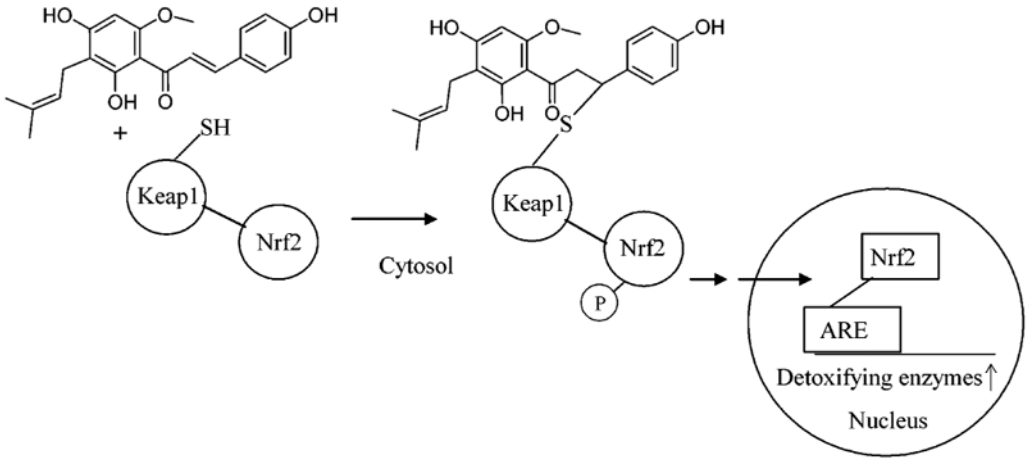
(A) Positive MALDI-TOF mass spectra of Keap1 (11 μM) modified by incubation with different concentrations of XH. (B) Number of modifications of Keap1 by XH at different ratios of XH to Keap1.



Scheme 1.

Redox Cycling of Menadione Inducing Oxidative Damage or Detoxification by QR[a]

[a] Mechanism of chemoprevention: 1, antioxidants; 2, quinone reductase.



Scheme 2.

Proposed Mechanism of QR Induction by XH through the Keap1–Nrf2 Pathway[a]

[a] Besides Keap1, the mitogen-activated protein kinase (MAPK) (53, 54), the protein kinase C (PKC) (55), and the phosphatidylinositol 3-kinase (PI3K) pathway (56, 57) play a role in the regulation of detoxification enzymes (17). PKC phosphorylation of serine-40 in Nrf2 is necessary for Nrf2 release from Keap1 (55). Within the nucleus, Nrf2 binds to the ARE as a heterodimer with either small Maf proteins, FosB, c-Jun, or JunD (46, 58). These proteins are omitted in the scheme for clarity.

Table 1.

Activity of a Hop Extract and Isolated Compounds in the DPPH, QR, and Cytotoxicity Assays

	compound	DPPH % FRS[a] ± SD	QR assay CD[c] (μM) ± SD	cytotoxicity assay LC_{50} (μM)
	hop extract[b]	38.4 ± 2.8 prenylated chalcones	3.2 ± 0.3[b]	> 20
1	XH	10.2 ± 4.1	1.7 ± 0.7 (2.1)[e] (1.7 ± 0.2)[f]	30.7 ± 7.6 (7.4 ± 1.4)[f]
2	1″,2″-dihydroxanthohumol K	−1.6 ± 3.0	2.5 ± 0.9	>20
3	desmethylxanthohumol B	9.7 ± 0.2	2.7 ± 0.1	>20
4	XH B	−3.1 ± 1.5	4.5 ± 0.6	>20
5	XH G	−2.4 ± 2.0	4.5 ± 0.2	>20
6	XH C	−1.1 ± 1.5	4.8 ± 2.4	>20
7	diprenylxanthohumol	12.1 ± 1.5	5.1 ± 1.2	>20
8	XH H	−2.1 ± 1.3	7.1 ± 0.7	>20
9	XH D	3.6 ± 2.9	7.4 ± 0.7	>20
10	desmethylxanthohumol J	3.4 ± 0.9	7.8 ± 0.6	>20
11	5″-*trans*-hydroxyxanthohumol	NA[d]	10.2 ± 0.6	>20
12	XH I	−4.9 ± 1.8 flavanones	>20	>20
13	6,8-diprenylnaringenin	17.4 ± 0.1	8.1 ± 0.5, (4.4)[e]	>20
14	isoxanthoflavan-3-ol	−3.3 ± 1.1	>20	>20
15	8-prenylnaringenin	2.9 ± 3.7	>30	>20
16	6-prenylnaringenin	1.7 ± 5.0	>30	>20
17	isoxanthohumol	3.3 ± 4.0	>50 (6.5 ± 1.5)[f]	30.7 ± 2.9

[a] % FRS represents the percent of free radicals scavenged at a screening concentration of 200 μM. Gallic acid was used as a positive control (IC_{50} = 35 μM). The IC_{50} is consistent with literature data (52).

[b] The hop extract ($CHCl_3$ fraction) was screened at a concentration of 200 μg/mL in the DPPH assay. Values are the means ± SD of triplicate determinations.

[c] CD value represents the concentration required for 2-fold induction of QR. The CD value was determined by at least two experiments performed independently with duplicate measurements. If CD > 20 μM, it was deemed inactive. BF (CD = 0.03 ± 0.02 μM) was used as a positive control in the QR assay; its CD value was consistent with literature data (8). The purity of all compounds was tested by qNMR and LC-MS.

[d] The compound was not tested in the DPPH assay, due to lack of sufficient material.

[e] Literature data for QR induction of XH and 6,8-diprenylnaringenin tested in Hepa 1c1c7 cells (20).

[f] Literature data for XH and isoxanthohumol ($n = 2$, QR induction measured in Hepa 1c1c7 cells; the effect on cellular growth was estimated by CV staining) (8).