Black Cohosh (*Cimicifuga racemosa* L.) Protects against Menadione-Induced DNA Damage through Scavenging of Reactive Oxygen Species: Bioassay-Directed Isolation and Characterization of Active Principles

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The roots/rhizomes of *Cimicifuga racemosa* L. (Nutt.) (black cohosh) have traditionally been used to treat menopausal symptoms through an unknown mechanism of action. In an effort to determine if black cohosh had additional health benefits, methanol extracts were investigated for their potential to scavenge reactive oxygen species and to protect against menadione-induced DNA damage. These extracts effectively scavenged 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals. In addition, the extracts showed dose-dependent decreases in DNA single-strand breaks and oxidized bases induced by the quinone menadione using the comet (single-cell gel electrophoresis assay) and fragment length associated repair enzyme assays, respectively. Bioassay-directed fractionation of the methanolic extracts using the DPPH assay as a monitor led to the isolation of nine antioxidant active compounds: caffeic acid (**1**), methyl caffeate (**2**), ferulic acid (**3**), isoferulic acid (**4**), fukinolic acid (**5**), cimicifugic acid A (**6**), cimicifugic acid B (**7**), cimicifugic acid F (**8**), cimiracemate A (**9**), and cimiracemate B (**10**). Six of these antioxidants were found to reduce menadione-induced DNA damage in cultured S30 breast cancer cells with the following order of potency: methyl caffeate (**2**) > caffeic acid (**1**) > ferulic acid (**3**) > cimiracemate A (**9**) > cimiracemate B (**10**) > fukinolic acid (**5**). These data suggest that black cohosh can protect against cellular DNA damage caused by reactive oxygen species by acting as antioxidants.

KEYWORDS: *Cimicifuga racemosa* (black cohosh, *Actaea racemosa*); single-cell gel electrophoresis assay (comet assay); menadione; antioxidant; reactive oxygen species

INTRODUCTION

Cimicifuga racemosa L. (Nutt.) (syn. *Actaea racemosa* L.) (black cohosh) from the plant family Ranunculaceae is native to the eastern United States and Canada. The roots/rhizomes have been used traditionally for menstrual symptoms and climacteric changes (*1*, *2*). In Japanese traditional medicine, the rhizome of *Cimicifuga* species is used for the treatment of inflammation (*3*). Black cohosh herbal preparations have also been utilized to reduce the frequency and intensity of hot flashes associated with menopause (*4*, *5*). Triterpene glycosides, aromatic acids, and their derivatives represent the main classes of compounds that have been isolated from the roots/rhizomes of *C. racemosa* (*6*–*12*). Although the aromatic acids, ferulic and caffeic acids, are well-known antioxidants, their biosynthetic derivatives isolated from black cohosh have not been evaluated for their ability to scavenge reactive oxygen species (ROS).

Free radicals and other ROS are constantly generated in vivo and cause oxidative damage to biomolecules. This process is regulated by the existence of multiple antioxidants, DNA repair systems, and the replacement of damaged lipids and proteins (*13*, *14*). The scavenging of ROS can protect against chronic diseases such as cancer, aging, and heart disease (*15*, *16*). However, increases in oxidative stress can potentially overwhelm repair systems and lead to cellular damage. DNA is a significant target of oxidative stress, because continuous oxidative damage contributes to the age-related development of colon, breast, rectum, and prostate cancers (*17*, *18*). This may be due

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to effects on cellular proliferation, prevention of apoptosis, damage to DNA repair enzymes, and damage to DNA polymerases that can decrease the fidelity of replication (*19*−*22*). Accordingly, if direct damage to DNA bases caused by ROS contributes to the development of cancer, agents that reduce such damage should decrease the risk of cancer development (*23*). As a result, in the current study we sought to establish if extracts of black cohosh could scavenge ROS and to identify the specific compounds responsible for the antioxidant activity.

Detection of compounds with antioxidant activity in complex plant extracts requires rapid and accurate methods. The stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) is the basis of a simple nonenzymatic assay to detect antioxidant compounds (*24*) that was used to direct the isolation of pure active compounds from black cohosh methanol extracts. To establish if black cohosh extracts and the isolated compounds could protect cellular DNA from single-strand breaks and oxidation of DNA bases, S30 breast cancer cells were treated with the prooxidant menadione, a quinone known to cause oxidative stress in cells (*25*). The extent of oxidative DNA single-strand breaks mediated by menadione in the presence of extracts and pure compounds was assessed using the comet assay (single-cell gel electrophoresis assay) (*26*). The level of oxidized DNA bases was evaluated using the fragment length associated repair enzyme (FLARE) assay, where DNA was exposed to the DNA repair enzyme formamidopyrimidine−DNA−glycosylase (Fpg). It was found that black cohosh contains antioxidants, mainly derived from hydroxycinnamic acids, and these agents provide protection from menadione-induced DNA damage.

MATERIALS AND METHODS

Materials and Reagents. All chemicals and reagents were obtained from Fisher Scientific (Hanover Park, IL) or Sigma (St. Louis, MO) unless otherwise indicated. Supplies for cell culture were obtained from Invitrogen Corp. (Carlsbad, CA). The roots and rhizomes of *C. racemosa* (L.) Nutt. were collected in Rockbridge County, Virginia (June 1999) (GPS coordinates 37° 48.27′ N × 79° 18.67′ W) and identified by Dr. G. Ramsey, Department of Biology, Lynchburg College, Lynchburg, VA. Voucher specimens have been deposited at the Ramsey−Freer Herbarium at Lynchburg College, Lynchburg, VA, and at the Field Museum of Natural History Herbarium, Chicago, IL. Caffeic acid, a compound known to be present in *C. racemosa* (*6*), was purchased from Sigma.

Instrumentation. NMR spectra were recorded with Bruker Avance 500 or Avance 300 spectrometers, operating at 500.160 or 300.130 MHz for ^{1}H and at 125.765 or 75.468 MHz for ^{13}C NMR, using CD$_3$-OD or pyridine-d_5 and TMS as an internal standard. Distortionless enhancement by polarization transfer (DEPT) experiments were performed using a transfer pulse of 135° to obtain positive signals for CH and CH$_3$ and negative signals for CH$_2$. ^{1}H−^{13}C heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond correlation (HMBC) experiments were performed using the standard Bruker software. Absorption spectra were determined with a Beckman UV spectrophotometer using methanol (MeOH) as a solvent. High-resolution electrospray ionization mass spectrometry (ESIMS) in the negative or positive ion mode was performed using a Micromass (Manchester, U.K.) quantitative time-of-flight (QTOF2) hybrid quadruple mass spectrometer. The samples were dissolved in MeOH and infused into the electrospray ion source using a syringe pump. MS-MS spectra were obtained using collision-induced dissociation at 12 eV with argon as the collision gas. Optical rotations were determined with a Perkin-Elmer 241 polarimeter equipped with a sodium lamp (589 nm) and a 10 cm microcell.

Chromatographic Systems. Thin-layer chromatography plates precoated with Kieselgel 60 F$_{254}$ Si and RP-18 (0.25 mm, E. Merck) were obtained from VWR (Bridgeport, NJ). Silica gel (mesh 230−400) chromatographic columns were eluted with CHCl$_3$/MeOH (gradient) or CHCl$_3$/acetone/H$_2$O (17:1:0.1), and 310 × 25 mm i.d., 40−63 μm, Lobar Lichreprep C$_{18}$ chromatographic columns (Merck, Whitehouse Station, NJ) were eluted with MeOH/H$_2$O (55:45) at a flow rate of 4 mL/min. UV detection was at 254 nm. Semipreparative HPLC was carried out with a Waters 996 system equipped with a photodiode array detector on a 300 × 20 mm, 5 μm, Watrex GROM-Si 120 ODS-4 HE semipreparative column (Watrex-International, Pittsford, NY) at a flow rate of 6 mL/min.

Extraction and Isolation. The air-dried, milled roots/rhizomes of *C. racemosa* (8 kg) were exhaustively extracted by percolation with MeOH followed by extraction with MeOH/H$_2$O (6:4) to afford extracts A (1250 g) and B (112 g), respectively. The methanol extract (A) was fractionated by successive partitions with ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH). Chromatographic separation of a portion (250 g) of the EtOAc fraction on silica gel led to eight subfractions. Workup of subfraction 4 (2.3 g) through a series of normal-phase silica gel columns, C$_{18}$ low-pressure column chromatography and semiprepative HPLC yielded isoferulic acid (**4**, 232 mg), ferulic acid (**3**, 42 mg), methyl caffeate (**2**, 12 mg), cimiracemates A (**9**, 8 mg), B (**10**, 5 mg), C (2.1 mg), and D (1.8 mg) as previously described (*12*). A sample of the MeOH/H$_2$O (6:4) extract (B, 112 g) was dissolved in water, chromatographed on a Diaion HP-20 column, and developed successively with H$_2$O, 20% MeOH, 50% MeOH, and 100% MeOH to yield fractions 1−4. Fraction 2 (20% MeOH, 5 g) was chromatographed on a polyamide column and eluted with MeOH and MeOH/H$_2$O (1:1) to afford two subfractions, 2A and 2B. Subfraction 2B (400 mg) was chromatographed on a semipreparative C$_{18}$ HPLC column employing a gradient solvent system of CH$_3$CN/H$_2$O (15:85 to 45:55 over 40 min, flow rate = 5 mL/min) to yield pure fukinolic acid (**5**, 13 mg) (*27*). Fraction 3 (50% MeOH fraction, 5 g) was chromatographed on a C$_{18}$ Lichreprep column and eluted with a MeOH/H$_2$O gradient from 1:2 to 1:0 to give crude cimicifugic acid A (34 mg), cimicifugic acid B (64 mg), and pure cimicifugic acid F (**8**, 8 mg) (*6, 28*). Cimicifugic acids A and B were further purified on a semipreparative C$_{18}$ HPLC column, eluting with isocratic solvents. Cimicifugic acid A (**6**, 14 mg) was obtained by elution with a mixture of CH$_3$CN/H$_2$O (25:75) acidified with 0.1% acetic acid (flow rate = 6 mL/min over 25 min), whereas cimicifugic acid B (**7**, 17 mg) was obtained by developing the column with CH$_3$CN/H$_2$O (27:73) acidified with 0.1% acetic acid. The identities of the isolates were established by comparison of their physical data, ^{1}H and ^{13}C NMR spectral data, and exact mass with those reported in the literature (*6, 27, 28*).

Cell Culture Conditions. The S30 cell line, a stable estrogen receptor alpha (ERα) transfectant of human MDA-MB-231 breast cancer cells, was provided by V. C. Jordan (Northwestern University, Chicago, IL). Cells were maintained in phenol red-free minimal essential medium with Earle's salts supplemented with 1% penicillin−streptomycin, 6 μg/L insulin, 500 mg/L G418 (geneticin disulfate salt), 1% Gluta-Max, and 5% charcoal/dextran twice-stripped fetal bovine serum (Atlanta Biologicals, Atlanta, GA). The cells were grown at 37 °C and 5% CO$_2$.

Comet Assay. The single-cell gel electrophoresis assay was carried out according to the procedure of the manufacturer with minor modifications (Trevigen, Gaithersburg, MD). S30 cells were plated and grown for 24 h prior to treatment with samples. After samples had been added, cells were incubated for 3 h and then treated with menadione for 30 min. 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 (75 μL) were immediately pipetted onto CometSlides (Trevigen, Gaithersburg, MD). The slides were then immersed into cold 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 40 min at 4 °C. After lysis, horizontal electrophoresis was performed at 4 °C for 20−40 min at 25 V and 300 mA. Slides were dried and stained with 50 μL of SYBR Green and viewed under a fluorescent microscope. The DNA was scored from 0 (intact DNA) to 4 (completely damaged DNA with tail only). Scores were calculated using the following formula in which N_A, N_B, N_C, N_D, and N_E were the number of cells demonstrating different comet tails from intact nuclei (N_A) through completely

Table 1. Scavenging of DPPH Free Radicals by Black Cohosh Methanol Extract and Pure Compounds[a]

extract or compound	IC$_{50}$ (μM)	% yield[b]
CH$_3$OH black cohosh extract	99 ± 5.0[c]	100
caffeic acid (**1**)	33.9 ± 2.4	not isolated
methyl caffeate (**2**)	53.1 ± 1.6	0.002
ferulic acid (**3**)	120 ± 3.9	0.005
isoferulic acid (**4**)	191 ± 2.0	0.03
fukinolic acid (**5**)	14.1 ± 0.11	0.001
cimicifugic acid A (**6**)	27.7 ± 4.0	0.0008
cimicifugic acid B (**7**)	22.9 ± 2.9	0.005
cimicifugic acid F (**8**)	20.5 ± 3.1	0.0008
cimiracemate A (**9**)	20.3 ± 2.4	0.001
cimiracemate B (**10**)	20.9 ± 4.5	0.0006
gallic acid[d]	4.92 ± 0.021	not isolated

[a] Values are the means ± SD of triplicate determinations. Experimental details are given under Materials and Methods. [b] Percent yield calculation based on the dry weight of the MeOH extract. [c] Methanol extract units are represented as μg/mL. [d] Positive control.

damaged DNA (N_E). Scores (S) = (I)/($N_A + N_B + N_C + N_D + N_E$) × 100. Triplicate samples were prepared for each treatment, and at least 100 cells were scored for each sample.

Oxidative DNA Damage Comet Assay. Instructions provided with the Trevigen Fpg FLARE kit were followed (Trevigen). Following a 3 h treatment with plant extracts and a 30 min incubation with menadione, cells were collected and washed as described above and suspended in cold PBS (1 × 10^5 cells/mL). An aliquot (50 μL) was combined with 1% low-melting agarose and pipetted onto Trevigen CometSlides. After the cell/agar mixture had been fixed to the slide, the cells were lysed at 4 °C in lysis buffer (described above) for 15 min. The slides were incubated for 60 min at 37 °C with the Fpg enzymes, and appropriate buffer-only controls were included. Following equilibration with alkali solution (500 mM EDTA adjusted with 5 M NaOH to pH 12.5), the slides underwent electrophoresis for 3 min at 1 V/cm. The slides were then fixed with 70% ethanol, stained with SYBR Green, and scored as described above. The difference between the Fpg-treated samples and the buffer controls was proportional to the amount of oxidative damage to DNA bases.

DPPH Assay. Reaction mixtures containing test samples (5 μL dissolved in DMSO or water) and 95 μL of a 300 μM DPPH ethanolic solution were incubated at 37 °C for 30 min in 96-well microtiter plates. Absorbance of the free radical abstracted 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 or water-treated control groups. All experiments were performed in triplicate, and IC$_{50}$ values were determined. IC$_{50}$ denotes the concentration of sample that was required to scavenge 50% of the DPPH radicals. Results are the mean ± standard deviation of three determinations.

Cytotoxicity Assay. S30 cells (4000/well) were preincubated in 96-well microtiter plates overnight in 200 μL of medium. The cells were then incubated with samples for 3 h, because this was the same amount of time the cells were preincubated with plant extracts to measure protection. Cells were then rinsed, fixed with 20% ice-cold trichloroacetic acid (TCA) overnight at 4 °C, stained with 0.4% sulforhodamine B (SRB) dissolved in 1% acetic acid for 30 min, and dried. The excess dye was rinsed four times with 1% acetic acid and dried. The dyed cellular protein was resuspended in 0.1 M Tris buffer (200 μL) with shaking, and the absorbance was measured at 515 nm. Percent growth was calculated as follows: [(absorbance$_{sample}$)/(absorbance$_{DMSO}$)] × 100 = % cell growth. Each sample was tested in triplicate.

RESULTS

Bioactivity-Guided Isolation. The crude methanol extract of the black cohosh roots/rhizome was shown to scavenge DPPH free radicals with an IC$_{50}$ of 99 μg/mL (**Table 1**). To identify the compounds in this plant extract responsible for the antioxi-
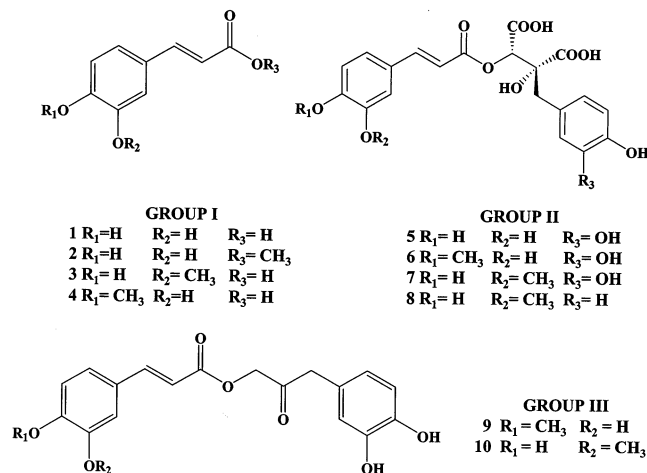

Figure 1. Compounds isolated from *C. racemosa*: (**1**) caffeic acid; (**2**) methyl caffeate; (**3**) ferulic acid; (**4**) isoferulic acid; (**5**) fukinolic acid; (**6**) cimicifugic acid A; (**7**) cimicifugic acid B; (**8**) cimicifugic acid F; (**9**) cimiracemate A; (**10**) cimiracemate B.

dant activity, bioassay-directed isolation was conducted to obtain compounds having the ability to scavenge 50% of the DPPH radicals at a concentration of ≤150 μg/mL. Workup of a subfraction of the EtOAc fraction prepared from the MeOH extract (A) of the roots/rhizomes led to the isolation of the phenylpropanoids, ferulic (**3**) and isoferulic (**4**) acids, methyl caffeate (**2**), and cimiracemates A (**9**), B (**10**), C, and D. A lack of sufficient material precluded the biological evaluation of the cimiracemates C and D. Similar workup of the MeOH/H$_2$O/ 6:4 extract (B) yielded the phenylpropanoid esters, fukinolic acid (**5**) and cimicifugic acids A (**6**), B (**7**), and F (**8**). Caffeic acid (**1**), the biosynthetic precursor of methyl caffeate, previously reported from this plant (*6*), was acquired commercially and also tested.

Compounds Isolated from Black Cohosh Scavenging DPPH Radicals. Free radical scavenging is commonly regarded as a mechanism responsible for protecting DNA against oxidative damage. The antioxidant activities observed with the black cohosh methanol extract and isolates were measured according to their potency to scavenge DPPH free radicals. The results are summarized in **Table 1**. Gallic acid (3,4,5-trihydroxybenzoic acid) was used as a positive control (*24*). In general, compounds in groups II and III had higher antioxidant activity then compounds in group I (**Figure 1**), probably due to the additional catechol ring.

Menadione-Induced DNA Single-Strand Breaks and Oxidized DNA Bases. Quinones such as menadione are potent redox active compounds that can undergo redox cycling with the semiquinone radical generating superoxide radicals mediated through cytochrome P450/P450 reductase (as shown in **Figure 2** for menadione). The reaction of superoxide anion radicals with hydrogen peroxide, formed by the enzymatic or spontaneous dismutation of superoxide anion radicals, in the presence of trace amounts of iron or other transition metals gives hydroxyl radicals, which cause single-strand DNA breaks and oxidation of DNA bases. Using the comet assay, we showed that increasing concentrations of menadione caused a dose-dependent enhancement in DNA single-strand breaks in S30 cells reaching a maximum at 10 μM menadione (**Figure 3A**). Doses exceeding 10 μM caused a reduction in cell viability (data not shown). All subsequent comet assays were performed with 10 μM menadione for 30 min to produce DNA strand breaks without generating cytotoxic effects.
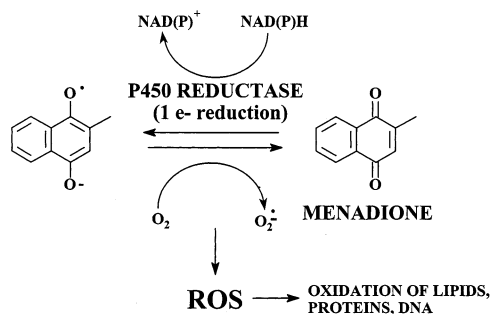
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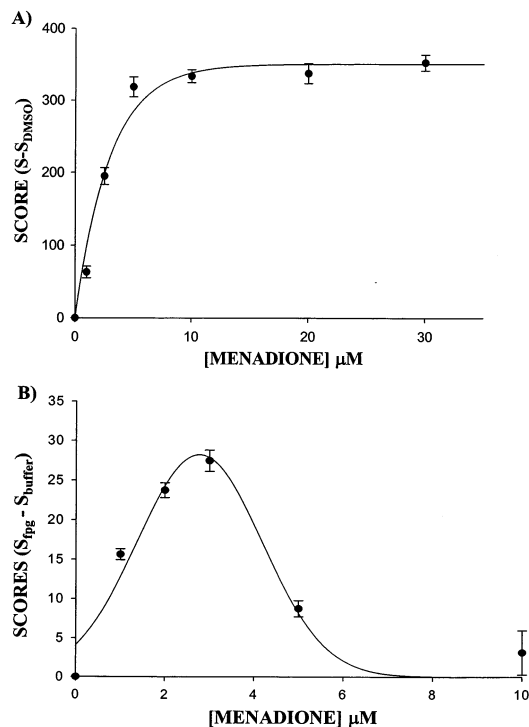**Figure 2.** Redox cycling of menadione inducing oxidative damage to cellular macromolecules.

Figure 3. Menadione-induced DNA damage in S30 breast cancer cells: (A) single-strand breaks measured using the alkaline single-cell gel electrophoresis assay [cells (3×10^5 cells/mL) were treated with menadione or vehicle for 30 min at 37 °C; data represent the average ± SD of three determinations]; (B) oxidized DNA bases measured by Fpg FLARE assay [S30 breast cancer cells were incubated with menadione for 30 min; the level of increased strand cleavage detected above cells not treated with Fpg enzyme represents the oxidative damage caused by menadione; data represent the average ± SD of three determinations].

The ROS formed from menadione can oxidize DNA bases, producing thymine glycol, 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxo-dG), or 2,6-diamino-4-hydroxy-5-formamidopyrimidine (*29*). Oxidized bases can be detected using the FLARE assay by treating cells with the DNA repair enzyme Fpg, which reveals additional DNA strand breaks caused by excision of oxidatively damaged bases. Menadione at $1-3$ μM produced a dose-dependent increase in S30 DNA single-strand cleavage specifically caused by the removal of oxidized bases from the DNA by Fpg (**Figure 3B**). A dose of menadione (2 μM) was chosen for subsequent FLARE assays because this dose elicited the largest detectable difference between samples treated with menadione alone or treated with both menadione and Fpg. At menadione concentrations exceeding 3 μM the DNA single-strand breaks formed from menadione became so prevalent that
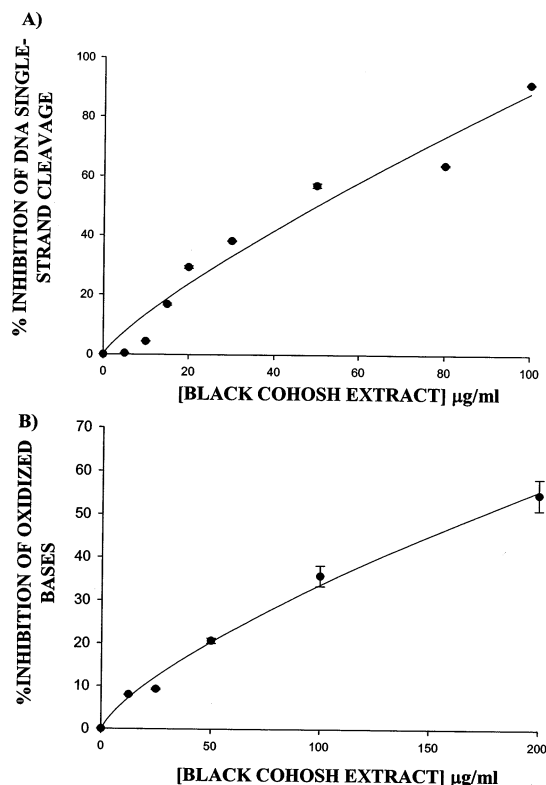

Figure 4. Inhibition of menadione-induced DNA damage by *C. racemosa* MeOH extract in S30 breast cancer cells: (A) inhibition of single-strand breaks after incubation with *C. racemosa* MeOH extract using the comet assay [S30 cells were incubated with the MeOH extract 3 h before exposure to menadione; each value is the average of triplicate measurements and the standard deviation]; (B) inhibition of oxidized bases from incubation with black cohosh MeOH extract using the FLARE assay [black cohosh MeOH extracts were preincubated with S30 cells for 3 h prior to exposure to menadione; each value represents triplicate measurements ± SD].

it masked the detection of additional breaks formed from the enzymatic excision of oxidized DNA bases in the FLARE assay (**Figure 3B**).

Black Cohosh Methanol Extract Protection of S30 Cellular DNA from Menadione-Induced Damage. The addition of the methanol extract of black cohosh to the cell culture medium 3 h prior to exposure to menadione protected S30 cellular DNA from single-strand breaks in a dose-dependent manner (**Figure 4A**). In addition, the methanol extract of black cohosh was capable of preventing oxidative damage to the DNA bases induced by 2 μM menadione using the FLARE assay (**Figure 4B**).

Compounds Isolated from Black Cohosh Inhibiting Menadione-Induced DNA Damage in the Comet Assay. Compounds that successfully scavenged DPPH radicals were tested in the comet assay to investigate their relative ability to protect cellular DNA from menadione-induced single-strand cleavage. In contrast to the results obtained from the DPPH assay, group I compounds produced the highest inhibition of DNA single-strand cleavage among those tested with the exception of isoferulic acid, which did not protect DNA (**Figure 5**). Fukinolic acid was the only compound in group II that provided any protection against menadione-induced DNA single-strand breaks. The antioxidant activities of cimiracemates A and B were similar to that of ferulic acid (**Figure 5**).

Antioxidant Compounds Isolated from Black Cohosh Not Cytotoxic in S30 Cells. Many phenolic compounds, which

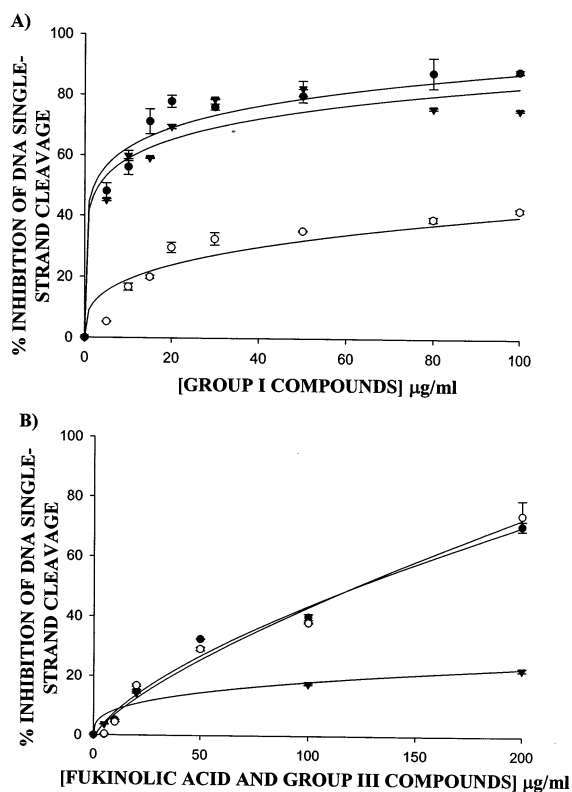**7026** *J. Agric. Food Chem.,* Vol. 50, No. 24, 2002

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Figure 5. Inhibition of DNA single-strand breaks induced by 10 μM menadione after incubation with pure compounds isolated from black cohosh using the comet assay: (A) inhibition of DNA single-strand breaks from group I compounds [each value is the average of triplicate measurements, and the standard deviation is noted by error bars; (●) methyl caffeate, (○) ferulic acid, (▼) caffeic acid]; (B) inhibition of DNA single-strand breaks after incubation with group III compounds and fukinolic acid [each value is the average of triplicate measurements, and the standard deviation is noted by error bars; (○) cimiracemate A, (●) cimiracemate B, (▼) fukinolic acid].

reduce oxidative stress by scavenging free radicals, can also undergo redox cycling and increase oxidative stress within the cell. For this reason, the potential toxic effects of the isolated compounds from black cohosh (**Figure 1**) were evaluated during a 3 h incubation at the highest dose tested in the comet assay (200 μg/mL) using the SRB assay (*30*). No compound showed significant reduction in cell growth compared to DMSO-treated cells (data not shown).

DISCUSSION

In the present study, menadione was used to induce oxidative damage and produce DNA single-strand breaks in S30 breast cancer cells as analyzed by the comet and FLARE assays. Menadione is a well-established inducer of oxidative damage known to result in extensive DNA single-strand cleavage and direct oxidative modification to the DNA bases resulting primarily in 8-oxo-dG (*31, 32*). As shown in **Figures 3**, menadione produced dose-dependent increases in DNA single-strand breaks and oxidized bases in S30 cells, which allowed for the selection of the optimal dose to test the potential of the black cohosh extracts and pure compounds to protect cellular DNA damage from reactive oxygen species. Similar studies have been carried out using hydrogen peroxide as the oxidant to screen for antioxidants in rosemary extracts (*33*). The black cohosh methanol extract was able to prevent both kinds of DNA damage induced by menadione; however, it was more effective

at reducing DNA single-strand breaks than reducing the amount of oxidized bases.

These data suggest that black cohosh extracts contain compounds that are effective scavengers of ROS that can protect cellular DNA against oxidative damage. Methyl caffeate, caffeic acid, ferulic acid, fukinolic acid, cimicifugic acid A, and cimiracemate B are all cinnamic acid derivatives that contain a phenol group para to the conjugated ethylene side chain. It is thought that the hydrogen on the phenol can be abstracted easily by a radical, such as DPPH or a hydroxyl radical, resulting in the formation of a phenoxy radical. This conformation is highly resonance stabilized because the unpaired electron can delocalize across the entire molecule (*34*). Among the compounds found to have antioxidant activity, those with the greatest number of phenolic groups tended to be the most effective free radical scavengers in the DPPH assay (**Table 1**). Structure–activity relationship studies have demonstrated that the presence of a second phenolic group allows for the formation of a catechol, which is important for antioxidant efficacy (*35*). The DPPH data currently reported are consistent with previous literature reports that showed the relative potency of hydroxycinnamic acid derivatives as effective antioxidants (*10, 36, 37*).

Among the compounds isolated from black cohosh with antioxidant behavior, methyl caffeate showed the highest potency in protecting DNA against single-strand cleavage with S30 breast cancer cells. The conversion of the acid group to the methoxy group decreases the polarity and might facilitate the transport of this compound across the cell membrane, where it can scavenge free radicals. Ferulic acid and caffeic acid also showed a dose-dependent reduction in DNA damage. It has been established that caffeic and ferulic acids protect DNA by reducing ROS (*38*). Ferulic acid is currently used therapeutically in sunscreens to protect against the ROS generated by UV, and caffeic acid has been recognized as an effective topical protection against UV radiation (*39*).

Hydroxycinnamic acid derivatives, similar to compounds in group III, have been proposed to protect against DNA damage by scavenging ROS (*40*). A number of other biological properties have been attributed to other hydroxycinnamic acid derivatives, and some of these have been identified as the active anti-inflammatory compounds in other species of *Cimicifuga.* Some of these biological effects reported from hydroxycinnamic acid derivatives include induction of the antioxidant response element mediating expression of NAD(P)H:quinone oxidoreductase, inhibition of both forms of cyclooxygenase (COX), reduction of activation of COX-2 gene expression, inhibition of lipoxygenase, reduction of rodent paw swelling induced by carrageenin, and inhibition of macrophage cytokines important in the inflammatory response (*3, 41−45*). The current results indicate that these types of compounds are also effective antioxidants. Cimiracemates A and B both reduced in a dose-dependent manner the single-strand DNA breaks induced by menadione in breast cancer cells.

In conclusion, we have shown that methanol extracts of *C. racemosa* L. contain antioxidant compounds, which have been isolated and characterized. Methyl caffeate, ferulic acid, and caffeic acid are the primary compounds responsible for the activity; however, minor components such as cimiracemate A and B may also contribute to the antioxidant activity of black cohosh. Reactive oxygen species are currently regarded as a major factor in the development of DNA mutations responsible for the progression toward certain cancerous lesions. Identifying new agents and new sources of antioxidant compounds may

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help provide protection against certain types of oxidation-related illnesses such as aging, cancer, and inflammation.

ABBREVIATIONS USED

DEPT, distortionless enhancements by polarization transfer; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ER, estrogen receptor; FLARE, fragment length analysis using repair enzymes; Fpg, formamidopyrimidine−DNA−glycosylase; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum coherence; QTOF, quantitative time-of-flight; ROS, reactive oxygen species; SRB, sulforhodamine B.

ACKNOWLEDGMENT

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